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13. ABSTRACT *(Maximum 200 Words)*

Directing the immune system to attack tumors represents a potential powerful approach to treat breast cancer. Our goals were to engineer the interleukin-2-receptor (IL-2R) in cytotoxic T cells (CTL) to control signal transduction through this receptor and to improve the in vivo efficacy of adoptive CTL immunotherapy. Although we produced a large number of chimeric IL-2R, we were unsuccessful to induce T cells activation through these chimeric molecules upon transfection into T cells. However, we establish a sensitive animal tumor model system to study the interaction of a solid tumor with naïve, effector and memory tumor-specific CTL. These studies indicate that naïve T cells are ignorant of the tumor, but upon immunization of tumor-bearing mice with tumor-antigen peptide pulsed antigen presenting cells, the naïve T cells were activated and mediated anti-tumor immune responses independent of CD4+ T helper cells. Similarly, adoptive transfer of CTL also inhibited tumor growth. Tumor-specific CTL memory T cells persisted in vivo for a long period after adoptive transfer in vivo provided the CTL were generated in vitro with IL-2. These memory CTL efficiently inhibited tumor growth suggesting that successful adoptive tumor therapy may depend on culture conditions that favor generation of memory CTL.

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Table of Contents

INTRODUCTION

IL-2 is an important cytokine that regulates a number of T cell activities, including promoting T cell growth, sensitizing T cells to activation-induced apoptosis, and enhancing CTL, LAK, and NK activity (1, 2). Early on, IL-2 showed much promise as an anti-tumor agent, but its use in patients has been problematic due to its short half-life and high toxicity in vivo. An important objective of this grant was to develop an approach in which the value of IL-2 as a therapeutic agent could be carefully evaluated in model systems without this toxicity and short half-life. Our plan was to exploit a small molecular weight drug, AP1510, that functions to dimerize signaling proteins by virtue of their specific binding to the FKBP-binding domain of immunophilin (3). This approach required the molecular construction of chimeric proteins consisting of the receptor or signaling protein of interests and the FKBP domain. These type of proteins have been successful utilized to induced signaling by cells surface receptors, intracellular signaling proteins, or transcription factors (4-12). We proposed that this approach might be applied to the IL-2R since initiation of its signaling cascade first depends on dimerization of its β and γc subunits (13, 14).

Our research maintained two parallel tracks. One was the molecular construction and testing of chimeric IL-2R that places their signaling under the control of the small synthetic drug, AP1510. The second was the development of a preclinical animal model that allowed us to evaluate the efficacy of such engineered IL-2R upon expression into tumor-specific T cells and their transfer to a tumor-bearing mouse. As will be outlined in the progress report, due to technical difficulties, we have been unsuccessful in preparing drug regulated IL-2R. However, we have developed and charactered in detail the immune responses to a model tumor antigen in this preclinical animal model. These studies were expanded, as approved in the revised statement of work, and suggest that the use of IL-2 in

vitro may have important benefits in the persistent of tumor reactive memory CTL. Thus, despite some of the technical difficulties, all our efforts have remained focused on the major objective of the grant, that is to better define conditions and approaches to improve the efficacy of adoptive T cell immunotherapy.

BODY

The following represents a summary of the research on this project. The results are summarized based on the original statement of work and necessary changes as outlined in the revised statement of work. Previously reported data are simply cited when contained in the annual or midterm progress reports. Most of our work in the preclinical animal model are described in detail in appended published or submitted manuscripts.

Technical objective 1: To develop a regulated drug-inducible IL-2R for expression in CTL.

As we reported in the first annual progress report, we prepared 14 chimeric IL-2R using conventional molecular biology techniques in which we varied the position and number of FKBP binding domains relative to the IL-2Rβ and γc cytoplasmic domains. 10 of the constructs are shown in Fig. 1. All these constructs were properly assembled as determined by restriction endonuclease mapping and sequencing. All of these chimeric molecules were readily expressed at high levels after transient transfection of the COS7 monkey kidney epithelial cell line using an episomal replicating vector. When the COS7 cells were further transfected with vectors containing cDNAs for Jak-1, Jak-3, and STAT5, only one of these chimeric pairs, pCMβF4E/pCMγF1E, induced tyrosine

phosphorylation of STAT5 when stimulated with AP1510. This level of STAT5 activation was somewhat lower than detected for control transfected COS7 cells that expressed the native IL-2R and were stimulated with IL-2. These data are contained in Fig. 2 of the First Annual Progress Report. However, after transfection of these constructs and others into an IL-2-responsive EL4 T cell tumor, we failed to induced STAT5 activation by AP1510. These data were shown in Fig. 1 of the Second Annual Progress Report. The difficulty in demonstrating signaling by AP1510 through these chimeric receptors necessitated us to prepare and test many more chimeric IL-2R constructs than we initially proposed. From these experiments, we concluded: 1) the orientation of the IL-2R cytoplasmic domains and FKBP domain are critical for activation by AP1510; 2) expression of these chimeric receptor is lower in T cells than COS7; and 3) that dimerization of the chimeric IL-2R is much less efficient that dimerization of native IL-2R by IL-2, with correspondingly much lower signal transduction.

In all the reported cases in which dimerization based on the FKBP domain was successful, the chimeric molecules showed biological activity due to homodimerization (4-12). So far, there are no reported cases in which this approach has been successful for heterodimerization. Therefore, we designed and prepared a second set of chimeric constructs in which IL-2Rβ and γc cytoplasmic domains were alined in a single molecule (Fig. 2) so its activation depends on homodimerization. In one set of constructs, the Jak-1 binding region (S domain) was deleted such that the size of the cytoplasmic domain of the chimeric construct was identical to that of IL-2Rβ, yet still potentially provided Jak-3 signaling via the cytoplasmic tail of γc. Our plan was to first test the ckit chimeric versions , as we can verify their membrane localization and could use a ligand to ckit, i.e. stem cell factor (SCF), with proven capacity to efficiently induced homodimerization. These ckit/IL-2R

chimeric molecules were transfected into EL4 and the IL-2-dependent CTLL cells. Unfortunately, in no case was STAT5 activated by SCF in the transfected EL4 cells nor did SCF substantially induce proliferation by the transfected CTLL. In the latter case, SCF induced proliferation by CTLL that was on average on 3% of that induced by IL-2 after 24 hr, and importantly, SCF could not sustain any proliferation after 48 hr. In the case of the CTLL cells, we verified that some of these transfected cells expressed levels of the chimeric receptors that were essential the same as the endogenous native IL-2R. These expression data were reported in Fig. 4 of the Second Annual Progress Report. Therefore, the failed biological response can not be attributed to poor expression of the chimeric receptor. Thus, for these experiments we concluded that we could not convert IL-2R signaling into a homodimeric chimeric receptor.

Technical objective 2: To examine the capacity of defective retrovirus containing chimeric IL-2R to transduce mouse and human breast cancer specific CTL and to promote the growth of these CTL in vitro.

Due to the technical difficulties in technical objective 1, it was not possible to proceed with transduction of mouse and human tumor-specific CTL with defective retrovirus containing chimeric IL-2R. However, as it was only clear late in this project that we were not going to achieve this objective, in anticipation of doing technical objective 2, a more efficient retroviral transduction system was employed in the lab to optimize transduction of primary T cells. This system utilizes the Phoenix packaging cell lines (15, 16) that produced high titer virus stocks upon transient transfection of vectors that contains viral LTRs, a packaging signal, an EBV sequence for episomal replication, and

the cDNA of interest. The cloning site of the vector we employed (15) also contained an IRES, with down-stream expression of human CD2 and upstream expression of any gene of interest. The advantage of this vector is that transduced mouse cells can easily be enumerated or purified with anti-human CD2.

The effectiveness of system is illustrated in Fig. 3. 24 hr after transduction of the BW5147 T cell thymoma, over 75% of the cells expressed human CD2 (Fig. 3A). Furthermore, we were also able to efficiently transduce primary activated mouse T cells. 24 hrs after transduction of anti-CD3 stimulated spleen cells, 21.5% and 10.5% of CD4 and CD8 T cells, respectively, expressed human CD2 (Fig. 3B). Furthermore, in other studies, when purified activated CD8 T cells were transduced, over 50% expressed human CD2. Thus, this retroviral system provide a framework for introduction of any gene of interest into mouse and human CTL for adoptive immunotherapy.

Technical objective 3: To assess the growth, survival, effector activity, and anti-tumor efficacy of transduced tumor-specific CTL upon adoptive transfer to tumor-bearing mice.

Although we were not able to study the anti-tumor effect of genetically engineered CTL in vivo, we examined in depth the capacity of naive, effector and memory CTL to reject a solid tumor. Our animal model uses ovalbumin (OVA)-specific OT-I T cell receptor transgenic T cells (17) as tumor specific T cells, and OVA-transfected EL4 cells, that express OVA as a model tumor antigen. These cells are designated E.G7 (18) and were transplanted subcutaneously to form a solid tumor mass. Many of these experiments were directly in line with the initial proposal, as we required a preclinical animal model to test the efficacy of CTL, with or without expression of chimeric

receptors. This aim was somewhat expanded as described in the revised statement of work, in part to quantify the fate and function of adoptively transferred CTL and to examine the extent that cytokines used to generate CTL in vitro affected their subsequent survival in vivo.

Most of these studies are described in manuscripts appended to this report. Appended manuscript 1 illustrates the fate and function of adoptive transferred of naive and effector CTL. Appended manuscript 2 examines the survival and subsequent function of adoptive transferred effector/memory CTL. These latter studies demonstrate that CTL generated under the appropriate culture conditions persisted in vivo for an extended period of time. These CTL acquired a memory phenotype and were competent to reject a solid tumor challenge. This latter result is very important within the scope of this proposal as it suggests that genetic engineering of CTL may in fact not be required to improve CTL efficacy for tumor therapy. It may simply require improved methods to rapidly generate CTL, in part, using the appropriate cytokines.

Although effector and memory OT-I CTL often prevented the growth of E.G7, in some mice this tumor eventually grew. Therefore, we also assessed the reason for these therapeutic failures. OT-I T cells were detected in mice that failed therapy (Fig. 4A) and these OT-I cell were not anergized as they readily proliferated to OVA in vitro (Fig. 4B). However, in each therapeutic failure where mice received a relatively high dose of OT-I CTL (2-10 x 10^6), the E.G7 cells recovered from these mice did not serve as targets for OT-I effector cells in CTL assays in vitro (Fig. 5A) and did not secrete a detectable level of OVA protein (Fig.5 B). These finding indicate that resistence of mice to E.G7 was secondary to selection of clones of this tumor that expressed low, immunologically undetectable, levels of the model tumor antigen. These data further illustrate a real limitation in CTL immunotherapy in which a single antigen is targeted, i.e. tumor antigen loss variants.

KEY RESEARCH ACCOMPLISHMENTS.

- A mouse model of tumor immunity has been developed in which a defined antigen that marked a solid tumor was rejected in a dose-dependent manner by the adoptive transfer of T cell receptor transgenic CD8 effector cells specific to this model tumor antigen or by immunization of tumor-bearing mice containing naive transgenic T cells with antigen-pulsed APC
- Mice adoptively transferred with relatively large number of naive tumor-specific transgenic T cells do not affect the growth of a solid subcutaneous tumor. This failed response was due to immunological ignorance, not an aborted immune response.
- Induction of an anti-tumor immune response by the CD8+ T cells occurred without CD4+ helper T cells.
- Mice adoptively transferred with OT-I effector CTL persist in vivo for an extended period of time and rejected a tumor challenge. The persistence and development of memory CTL phenotype in vivo was dependent upon the use of IL-2 during the priming of the effector CTL in vitro prior to adoptive transfer.
- Tumor antigen loss variants are a major reason for therapeutic failure by CTL that target a single tumor antigen.

REPORTABLE OUTCOMES

Abstracts

Dalyot-Herman, N, Malek, TR. Anti-tumor immunity of naive and activated class-I-restricted tumor-

specific TCR-transgenic T cells. Experimental Biology, 1999.

Dalyot-Herman, N. and Malek, T.R. Reversal of T cell ignorance and induction of anti-tumor immunity by peptide-pulsed antigen presenting cells. Era of Hope DOD Meeting, 2000.

Bathe, O.F., Dalyot-Herman, N. and Malek, T.R. Adoptive transfer of tumor-specific CD8+ effector/memory cells efficiently confers long-term protection against tumor inoculum. American Association of Cancer Research, 2001.

Bathe, O.F., Dalyot-Herman, N. and Malek, T.R. Emergence of antigen loss variants: limitation in efficacy following adoptive immunotherapy with cytotoxic T lymphocytes targeted at a single antigen. American Society Clinical of Oncologists, 2001.

Manuscripts.

Dalyot-Herman, N., Bathe, O. and Malek, T. R. Reversal of CD8+ T cell ignorance and induction of anti-tumor immunity by peptide-pulsed antigen presenting cells. *J. Immunol*: 165, 6731-6737, 2000

Bathe, O.F., Dalyot-Herman, N. and Malek, T.R. IL-2 during in vitro priming promotes subsequent engraftment and successful adoptive tumor immunotherapy by persistent memory phenotypic $CD8^+$ T cells[1]. (Submitted to *J. Immunol.* Revised and under reconsideration).

Employment from postdoctoral training under this award:

Nava Dalyot-Herman, Ph.D. Research Scientist, Sigma Israel Chemicals, Ltd., Jerusalem, Israel

Oliver Bathe, Assistant Professor, University of Calgary, Canada.

PERSONNEL ENGAGED IN PROJECT:

Received salary support:

N. Dalyot-Herman	Postdoc	7/98-8/00	100% effort
E. Codias	Res. Ass.	10/00-3/01	50% effort
R. Carrio	Postdoc	1/01-7/01	100% effort
A. Yu	Res. Tech.	6/99-5/00	50% effort
L. Kong	Res. Tech.	10/00-7/01	50% effort

Did not receive salary support:

T. Malek	PI	7/98-7/01	15% effort
O. Bathe	Postdoc	7/99-8/00	75% effort

CONCLUSIONS

The most important conclusion from our studies is that solid tumors such as breast cancer remain amenable to treatment by immunotherapy. In our mouse model system, we examined the potential of a relatively large number of tumor-specific naive T cells to respond against an antigen-marked tumor that was transplanted subcutaneously and growing as a solid mass. These naive tumor-specific T cells simply ignored the tumor, they were not anergized or actively suppressed. However, the anti-tumor activity of these naive T cells was unleashed simply by appropriate immunization, in

our case by using peptide-pulsed antigen presenting cells as a vaccine. With respect to adoptive immunotherapy, we found that once activated in vitro, adoptive transfer of effector CTL to a tumor-bearing animal resulted in an anti-tumor effect.

A major hypothesis for the initiation of this research was that there are ex vivo means to "engineer" CTL that will enhance their growth, survival, and effector function upon adoptive transfer to a tumor-bearing recipient, leading to improved anti-tumor efficacy. Therefore, perhaps the most exciting result from these studies is our finding that tumor-specific CTL generated in vitro are not in fact limited in their potential to persist in vivo after adoptive transfer and retained their cytolytic activity against the tumor. Long-term persistent of memory/effector CTL did not require genetic engineering or gene transfer, as we proposed and worked toward, but was dependent upon the cytokines used in the generation of the effector CTL with IL-2 being the critical factor. We reason that our success in programming CTL to acquire a persistent memory phenotype is two fold, programming by IL-2 and rapid generation of CTL in vitro for adoptive immunotherapy. Therefore, we now hypothesize that successful adoptive tumor immunotherapy requires adaptation of this approach for rapid production of tumor-specific CTL under condition that favors their development into memory CTL.

We never had the opportunity to directly test whether activation of the IL-2R could be placed under the control of a synthetic drug and then enhance the efficacy of CTL in tumor-bearing individuals. After extensive in vitro testing, we failed to generate a drug-regulatable IL-2R that supported the survival or growth of T cells in vitro. From this work we conclude that the spacial relationship between the signaling domains of IL-2Rβ and γc must strictly be maintained for proper signal transduction and subsequent T cell proliferation. However, our studies on the persistence and

function of CTL in vivo, as discussed just above, suggest that targeting the IL-2R signaling in vivo may not be the key limitation of adoptive T cell therapy. Our data suggest that the more important issues are to increase the frequency of tumor-reactive T cells, so effector cells may be adoptively transferred without the need for long-term cytokine driven CTL expansion, and to target multiple tumor-specific antigens.

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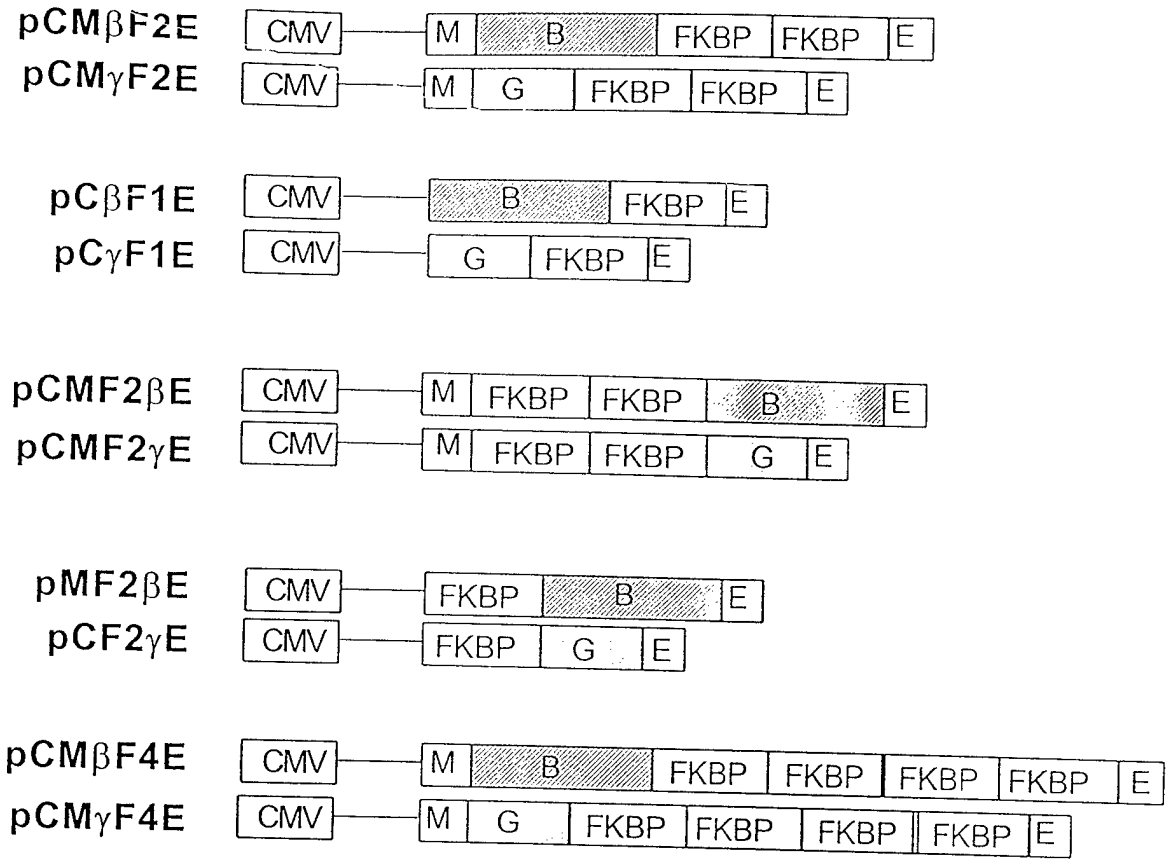


Fig. 1: Constructs of IL-2R drug induced chimeric molecules. The different plasmids were constructed by inserting the PCR fragments of IL-2Rβ and γ cytoplamic tails with SpeI site at the ends, to the vectors pCMF2E or pCF1E, using the XbaI site (for upstream) or the SpeI site (for downstream).
CMV- CMV promoter, M- Myristoylation signal, FKBP- binding domain for FKBP12 or AP1510, B- cytoplasmic tail of IL-2R Betta, G- cytoplasmic tail of IL-2R Gamma, E- HA epitope.

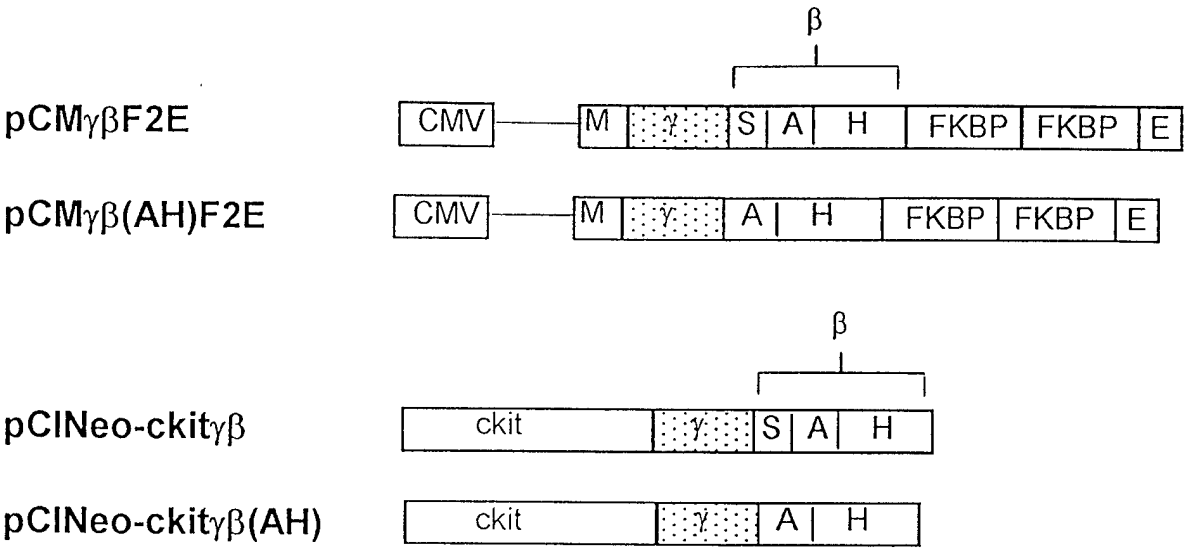
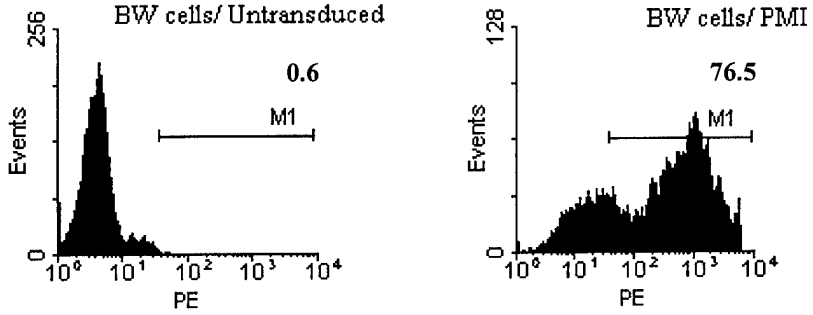


Fig. 2: Constructs of IL-2R chimeric molecules.
pCMγβF2E and pCMγβ(AH)F2E were constructed by inserting the PCR fragments of IL-2Rβ (S-A-H or A-H domaines, respectively) into the IL-2Rγ cytoplamic tail at the AvrII site using the SpeI site.
CMV,CMV promoter; M- Myristoylation signal; FKBP, binding domain for FKBP12 or AP1510; β- cytoplasmic tail of IL-2Rβ, γ, cytoplasmic tail of IL-2Rγc; E, HA epitope.
pCINeo-CKITγβ and pCINeo-CKITγβ(AH) were constructed by inserting PCR fragments of the extracellular and transmembrane domaine of CKIT (XhoI-EcoRI), IL-2Rγ cytoplamic tail (EcoRI-SalI) and IL-2Rβ cytoplamic tail (S-A-H or A-H domaines, respectively) (SalI-NotI) into the multiple cloning sites of pCINeo plasmid.

A



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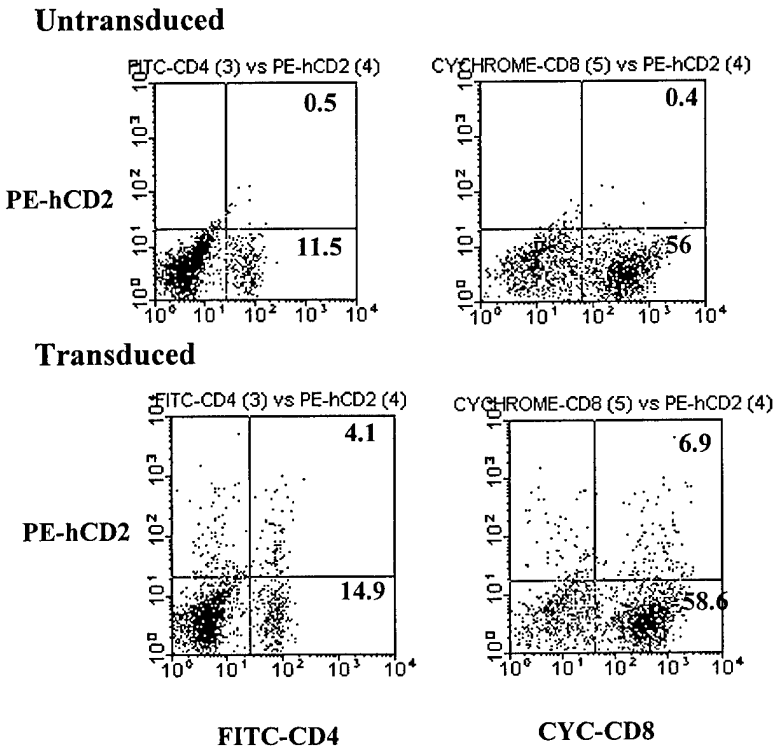

Fig. 3. Transduction efficiency of Mouse T cells. A. The BW5147 T cell thymoma was untransduced or transduced with pMI-human CD2 defective retrovirus and 24 hr later expression of human CD2 was assessed by FACS analysis. B. C57BL/6 spleen cells were activated with anti-CD3 for 24 hr and then left untransduced or transduced with pMI-CD2. 24 hr later the mouse T cells were examined for expression of human CD2 by FACS analysis. The number in the histograms or dot plots represent the % of fluorescent positive cells.

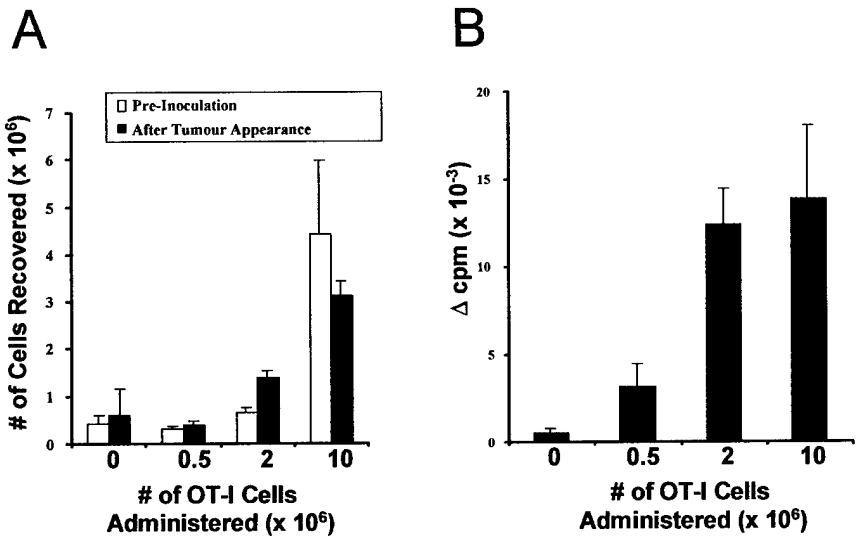

Fig.4. Properties of persistent donor cells at the time of tumor challenge (ie: day 21–28) and after appearance of tumor. A. Number of persistent donor cells recovered from spleen and 4 lymph nodes, as measured prior to tumor challenge and at the time of therapeutic failure. Donor cells were identified by staining for CD8, Vα2-TCR and Vβ5.1,5.2-TCR. B. Proliferative response of donor cells as determined by ^{3}H-thymidine uptake assay. Splenocytes from recipients of OT-I CTL were harvested after therapeutic failure and pulsed with ovalbumin peptide (5nM).

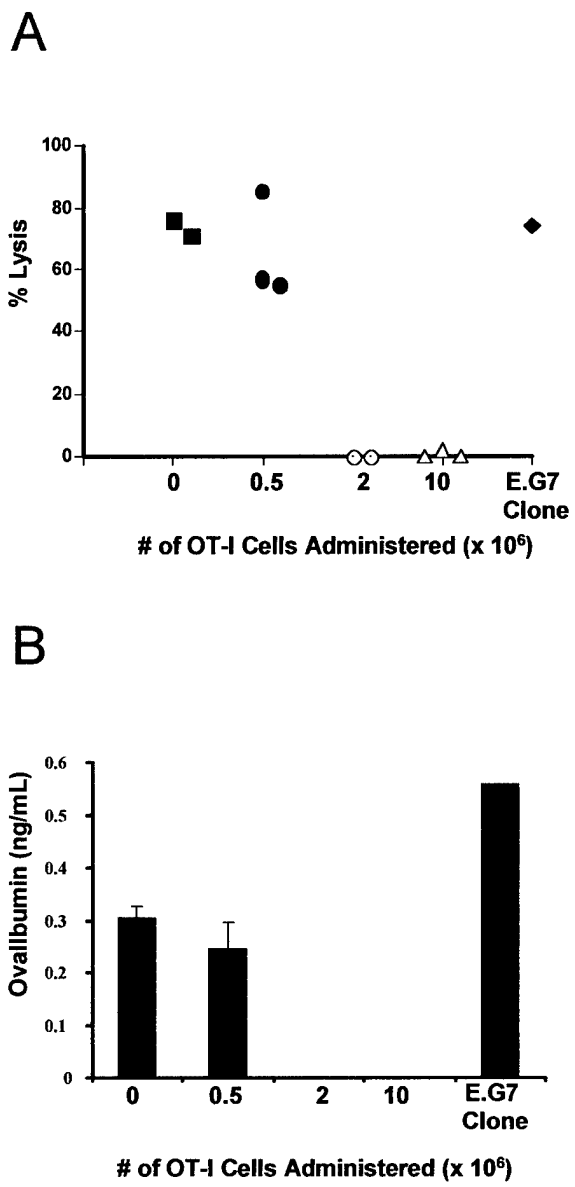

Figure 5. Analysis of tumors extracted from mice which failed to reject a tumor challenge after adoptive transfer of various numbers of OT-I CTL. A. Cytotoxicity of freshly generated OT-I CTL against E.G7 tumor derived from mice which developed tumor. Data are expressed as % cytotoxicity at an effector-to-target ratio of 100:1. B. Ovalbumin secretion by E.G7 tumors derived from mice which developed tumor. Tumor cells (1 x 10^6) from each individual were cultured in duplicates and supernatants were collected at 24 hours for ELISA determination of ovalbumin content.

Reversal of CD8$^+$ T Cell Ignorance and Induction of Anti-Tumor Immunity by Peptide-Pulsed APC[1]

Nava Dalyot-Herman,* Oliver F. Bathe,† and Thomas R. Malek[2]*

In the present report, we have studied the potential of naive and activated effector CD8$^+$ T cells to function as anti-tumor T cells to a solid tumor using OVA-specific T cells from TCR-transgenic OT-I mice. Adoptive transfer of naive OT-I T cells into tumor-bearing syngeneic mice did not inhibit tumor cell growth. The adoptively transferred OT-I T cells did not proliferate in lymphoid tissue of tumor-bearing mice and were not anergized by the tumor. In contrast, adoptive transfer of preactivated OT-I CTL inhibited tumor growth in a dose-dependent manner, indicating that E.G7 was susceptible to immune effector cells. Importantly, naive OT-I T cells proliferated and elicited an anti-tumor response if they were adoptively transferred into normal or CD4-deficient mice that were then vaccinated with GM-CSF-induced bone marrow-derived OVA-pulsed APC. Collectively, these data indicate that even though naive tumor-specific T cells are present at a relatively high fraction they remain ignorant of the tumor and demonstrate that a CD8-mediated anti-tumor response can be induced by Ag-pulsed APC without CD4 T cell help. *The Journal of Immunology,* **2000, 165: 6731–6737.**

It is now well-established that some tumors express Ags that result in induction of anti-tumor immune responses (1, 2). Although in some cases such a response results in eradication of the tumor, more often an anti-tumor immune response is ineffective, and the tumor ultimately grows. There are a number of specific mechanisms by which a tumor evades an ongoing immune response. These vary widely and include: development of tumor-Ag loss variants; down-regulation of surface MHC molecules or other molecules required for Ag presentation by the tumor cells (3, 4); induction of anergy of tumor-specific T cells due to lack of costimulatory molecules on the tumor cells (5–7); and suppression of the immune response by tumor secretion of inhibitory cytokines (8, 9). Tumor Ags by nature are usually dysregulated self-proteins or variants of self-Ags. Therefore, anti-tumor immunity may also fail due to a weak response to the Ags expressed by the replicating tumor or because potentially tumor-reactive T cells are tolerant to the tumor Ags.

Although the fate and function of adoptively transferred tumor-specific effector T cells have been extensively studied (10–12), comparatively little is known concerning the initial activation of tumor-specific T cells in vivo, primarily due to their low frequency in the peripheral lymphocyte pool. With the introduction of TCR-transgenic mice, this problem has been overcome. Analysis of an Ag-specific T cell response to nominal Ags is facilitated by adoptive transfer of a relatively low, but detectable, number of TCR-transgenic T cells to normal mice and then challenging such animals with the appropriate Ag (13). This approach allows direct phenotypic and functional characterization of the responding Ag-specific transgenic T cells during the course of the immune response and avoids the complications inherent in direct Ag stimulation of the TCR-transgenic mouse, in which all the T cells are Ag responsive.

More recently, this method has been adapted to study the induction of anti-tumor immunity in vivo. In this setting, a predetermined number of naive TCR-transgenic T cells are adoptively transferred to mice bearing a tumor that was transfected with an Ag recognized by the transgenic T cells (14–18). The present study employed this TCR-transgenic strategy to compare the capacity of naive and activated effector-transgenic CD8$^+$ T cells to generate an anti-tumor immune response to a solid tumor. We used OT-I TCR-transgenic T cells that are specific for $OVA_{257-264}$ peptide bound to H-2K^b (19) as the source of anti-tumor-specific T cells and the OVA-transfected EL4 cell line, E.G7, as the tumor cells expressing a tumor-specific Ag, i.e., OVA (20). We demonstrated that the naive OT-I T cells are functionally blind or ignorant of the OVA tumor Ag. This failure of OT-I T cells to respond to this tumor was overcome by proper Ag presentation, as supplied by peptide-pulsed professional APC, leading to an effective anti-tumor immune response. These findings provide a relevant strategy to overcome tumor Ag ignorance for cancer immunotherapy.

Materials and Methods

Animals

The OT-I TCR-transgenic mice (19) were maintained by breeding heterozygous OT-I TCR-transgenic mice to wild-type C57BL/6. The progeny mice were screened by PCR for the expression of the OVA-TCR gene. Six- to 10-wk-old female C57BL/6 or CD4-deficient mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME).

Cell lines

EL-4, a thymoma derived from the C57BL/6 mouse (H-2^b), was obtained from American Type Culture Collection (ATCC, Manassas, VA). OVA-transfected EL-4, designated as E.G7 (20), was a gift from Dr. M. Bevan (University of Washington, Seattle, WA). These cell lines were maintained in RPMI 1640 containing 5% FCS, glutamine (30 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2-ME (5×10^{-5} M) (complete medium).

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Abs and other reagents

OVA peptide (SIINFEKL) (21) was synthesized by Research Genetics (Huntsville, AL). Directly conjugated mAbs, including CyChrome-conjugated anti-CD8α, PE-conjugated anti-mouse Vα2 TCR, and FITC-conjugated anti-mouse Vβ5.1, 5.2 TCR, were purchased from PharMingen (San Diego, CA). CFSE was purchased from Molecular Probes (Eugene, OR). Cells were labeled with CFSE as previously described (22). Briefly, cells (2×10^7/ml) were incubated with 5 μM CFSE (from a 5 mM stock in DMSO) in serum-free medium for 10 min at 37°C and washed twice with cold complete medium and twice with HBSS.

Tumor challenge and adoptive transfer of transgenic T cells

Normal C57BL/6 or $CD4^{-/-}$ mice were injected with 1×10^6 E.G7 cells in 0.2 ml HBSS s.c. into the midline of the abdomen. The tumor cells were freshly thawed and grown in culture for 5–10 days before each injection. Spleen cell suspensions from heterozygous OT-I mice (>6 wk of age) were prepared as previously described (15). Splenocytes containing the indicated number of transgenic OT-I T cells were injected i.v. in 0.5 ml HBSS 5–7 days after tumor challenge.

FACS analysis

Spleens and draining lymph nodes (LN)[3] (inguinal, brachial) were collected and subjected to FACS analysis as previously described (15). Between 50,000 and 100,000 events per sample were collected on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CellQuest software (Becton Dickinson). OT-I-transgenic T cells were enumerated either by three-color staining for CD8, Vα2-TCR, and Vβ5.1, 5.2-TCR or by the fraction of CSFE-labeled (FITC) OT-I spleen cells that also stained for CD8. The latter approach is valid because essentially all $CD8^+$ cells in OT-I mice express the transgenic TCR.

Purification of $CD8^+$ OT-I cells

OT-I T cells were purified by a combination of negative and positive selection. First, B cells were depleted on anti-Ig-coated plates, followed by further depletion by incubation of the nonadherent cells with anti-CD24 (JIID), anti-NK1.1, and anti-MHC class II and C′ for 45 min at 37°C. The OT-I T cells were then subjected to positive selection using magnetic beads containing anti-CD8 (Miltenyi Biotec, Auburn, CA). The resulting cells were ≥95% pure as judged by FACS analysis.

T cell proliferation assay

T cells (2×10^5/well) were cultured in flat-bottom 96-well plates in complete medium containing the indicated stimuli for 3–4 days. EL4 or E.G7 cells were always irradiated (20,000 rad). Then 1 μCi [^{3}H]thymidine was added to the cultures for the last 5–6 h. The cells were harvested with an automated cell harvester, and the radioactivity incorporated in DNA was measured in a scintillation counter. Data of triplicate values that varied by <10% of the mean are displayed as Δcpm, i.e., cpm from experimental culture minus cpm from cultures containing only medium.

Culture for APCs

A single-cell suspension of bone marrow cells from normal C57BL/6 mice was cultured at 0.5×10^6/ml in complete medium containing 2 ng/ml murine GM-CSF (PeproTech, Rocky Hill, NJ). Four to 5 days later, adherent cells were collected after incubation with PBS containing 5 mM EDTA at 37°C for 15 min. The cells were washed with HBSS, incubated with 1 μM OVA peptide for 1 h at 37°C, and washed three times with HBSS. Mice were injected i.v. with the OVA-pulsed APC in 0.5 ml of HBSS.

Generation and assay of CTL

Splenocytes (1×10^6) from OT-I-transgenic mice were cultured in complete medium containing 1 nM OVA peptide, 20 U/ml IL-2, and 40 U/ml IL-4. After 3 days, the cells were collected, washed, and re-cultured at 1×10^5 cells/ml in complete medium containing 20 U/ml IL-2 and 40 U/ml IL-4 for 2 days. The CTL activity of the cells was measured by a standard ^{51}Cr release assay as previously described (23) using ^{51}Cr-labeled EL4 or E.G7 cells as the targets.

[3] Abbreviation used in this paper: LN, lymph node.

Results

Activation of OT-I in vitro

In this study, the OVA-specific MHC class I-restricted ($H2^b$) OT-I TCR-transgenic $CD8^+$ T cells were used as tumor-specific T cells by adoptive transfer to mice bearing the E.G7 tumor (OVA-transfected EL4) as a solid tumor. The presence of the OT-I cells in vivo was assessed by the coexpression of Vβ5 and Vα2 of the transgenic TCR on $CD8^+$ T cells. In our initial studies, we determined the potential to functionally measure low numbers of OT-I T cells in peripheral lymphoid tissue by evaluating the proliferative response to OVA peptide or E.G7 in vitro. Dose-response studies demonstrated that OT-I T cells developed strong proliferative responses to 10^{-7}–10^{-9} M OVA peptide, with detectable responses in cultures containing as little as 10^{-12} M OVA peptide (Fig. 1*B*). Furthermore, by mixing OT-I spleen cells with normal C57BL/6 spleen cells in such a manner that the fraction of transgenic OT-I cells was predetermined, readily detectable proliferative responses were routinely generated by a relatively high dose of OVA peptide (10^{-9} M) when the cultures contained as few as 1×10^3 OT-I T cells, which is only 0.5% of the total number of spleen cells in culture (Fig. 1*A*). Thus, OT-I cells are exquisitely sensitive to OVA peptide, and OT-I T cells were functionally detected in lymphoid tissues when present at a frequency of 1 in 200.

The E.G7 cells used in this study secreted 560 pg of OVA/ml/1×10^6 cells after a 24-h culture as determined by ELISA (data not shown). The addition of as much as 1.0 mg/ml of native soluble OVA protein induced only minimal proliferation of OT-I T cells in vitro (data not shown). Despite this relatively low level of

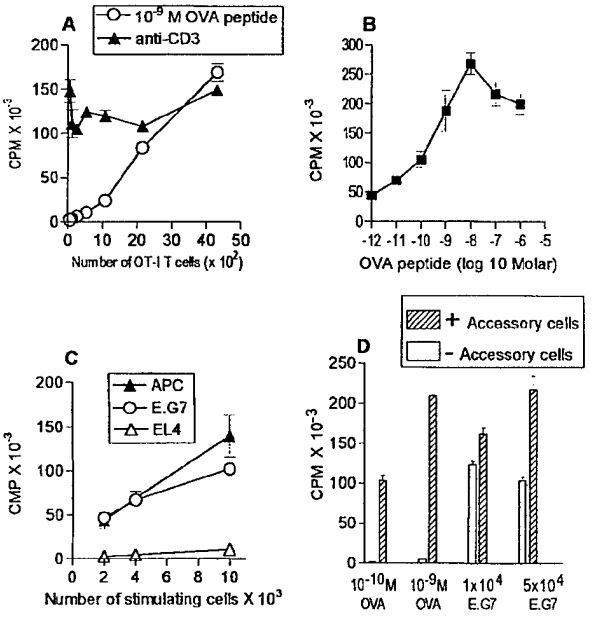

FIGURE 1. Response of OT-I cells in vitro. *A*, Response by limiting number of OT-I cells. *B*, Dose response to OVA peptide. *C*, Response to cell associated-OVA. *D*, Response by purified OT-I cells. C57BL/6 spleen cells (2×10^5/well) containing the indicated number of OT-I T cells (*A*) or 2×10^3/well OT-I T cells (*B* and *C*) were stimulated for 3 days with the indicated concentration of OVA peptide, anti-CD3 (5% supernatant), or the indicated number of irradiated EL4, E.G7, or OVA-pulsed APC. *D*, A total of 2.5×10^4 purified OT-I cells were stimulated for 3 days with the indicated concentration of OVA peptide or the indicated number of irradiated E.G7 in the presence or absence of 1×10^5 accessory cells. Data are representative of two to five experiments.

secretion of OVA, the E.G7 cells were immunogenic, as the irradiated E.G7, but not the parental EL4 thymoma, induced proliferation by OT-I cells in a fashion comparable to peptide-pulsed APC (Fig. 1*C*). Taken together, these data suggest that cell-associated OVA is much more immunogenic than soluble OVA protein. Furthermore, E.G7 directly stimulated substantial proliferation by highly purified OT-I T cells in the absence of accessory cells (Fig. 1*D*). By contrast, the response of these purified T cells to soluble OVA peptide was dependent upon accessory cells. These results indicate that the E.G7 can directly present OVA to OT-I.

OT-I T cells lack anti-tumor activity in vivo

Given the potent immunogenicity of E.G7 for OT-I T cells in vitro, we examined the ability of OT-I T cells to mount an anti-tumor immune response in vivo. Five days after C57BL/6 mice received 10^6 E.G7 s.c., OT-I T cells were adoptively transferred to these tumor-bearing mice, and tumor size was measured over time. Each mouse received 2.5×10^6 OT-I T cells, yielding mice in which ~5% of their $CD8^+$ T cells were OT-I. As reported by Jenkins and coworkers (13), this number of transgenic T cells seeds the peripheral immune compartment so that the transgenic T cells are present at a frequency that is still detectable by FACS analysis, but these cells are not at such a high level as to cause imbalance in the peripheral immune compartment. When compared with control mice that did not receive OT-I cells, it is quite apparent that rate of tumor growth was comparable between both groups of mice (Fig. 2). Transfer of a larger number (4×10^6) of OT-I T cells still failed to affect tumor cell growth (data not shown). Thus, OT-I cells did not generate as obvious an anti-tumor response to E.G7 growing as did a solid tumor.

To examine whether the adoptively transferred OT-I cells proliferated to E.G7 in vivo, we determined the proportion of OT-I T cells in the spleen and draining LN of tumor-bearing animals by three-color FACS analysis by staining for CD8, Vα2, and Vβ5. In a normal mouse, ~0.2% and ~0.5% of spleen and LN cells, respectively, express these three surface molecules. After adoptive transfer of OT-I T cells, the fraction of cells bearing these markers initially increased to ~0.6% for the spleen and ~1.3% for the draining LN (Fig. 3*A*). Importantly, these numbers decreased over time for both control and tumor-bearing animals to a level similar to that seen in normal mice. The total spleen and LN recovery, and the proportion of $CD8^+$ T cells from mice that were adoptively transferred with OT-I cells, in the presence or absence of E.G7, was always comparable (data not shown), further indicating that there was no obvious lymphoid cell expansion by the tumor. These findings suggest that OT-I T cells did not proliferate in response to the E.G7 tumor.

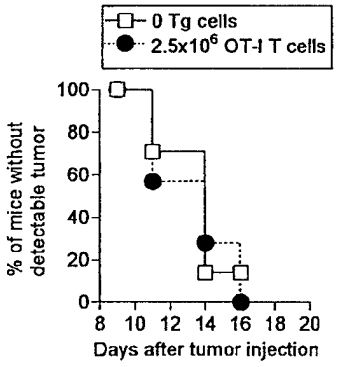

FIGURE 2. Anti-tumor activity of naive OT-I cells. Normal C57BL/6 mice were injected with E.G7 (1×10^6). Seven days later, mice received OT-I T cells as indicated, and the tumor size was measured. Detectable tumor is considered to be above 0.5 cm^2. The mice were sacrificed when the tumor reached a size of 2.0 cm^2. Data shown contain eight mice/group and are representative of three experiments.

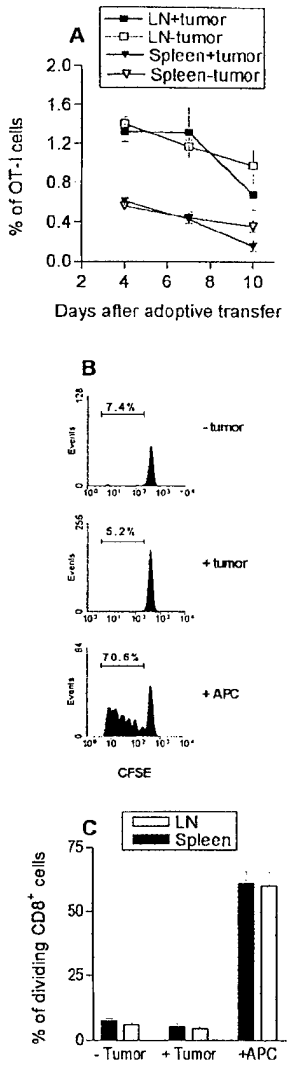

FIGURE 3. OT-I cells do not respond to E.G7 in vivo. *A*, Proportion of OT-I T cells in tumor-bearing mice. The percentage of Vα2, Vβ5, $CD8^+$ three-color-positive cells was determined for the spleens and LN of normal and tumor-bearing mice as described in *Materials and Methods*. Data shown contain four mice/group and is representative of three experiments. *B*, Cell division by OT-I cells. OT-I spleen cells were labeled with CFSE before transferring to normal or tumor-bearing mice (2.5×10^6). Some mice received 0.5×10^6 OVA-pulsed APC 1 day later. Three days after transfer of APC, LN cells were analyzed for cell division by two-color FACS analysis. Shown are histograms of cells double-stained for CD8 and CFSE. The percentage of cells that have divided, as seen by a reduction in CFSE staining intensity, is listed in *C*. Quantitative analyses of five to six mice in each group (mean ± SD), based on FACS histograms as shown in *B*.

To further evaluate whether OT-I cells responded to E.G7 in vivo, CFSE-treated OT-I T cells were adoptively transferred to E.G7-bearing mice. CFSE is an intracellular fluorescent label for which the fluorescence intensity decreases proportionally upon cell division (22). When CD8 cells in the draining LN and spleens of control and tumor-bearing mice were subjected to FACS analysis, the large majority of cells maintained the original fluorescence

intensity, a profile characteristic of cells that have not undergone cell division. Representative FACS histograms are shown in Fig. 3*B*, and the results from the analysis of multiple mice are summarized in Fig. 3*C*. Very few OT-I cells exhibited a decrease in CFSE staining after adoptive transfer to normal or E.G7-bearing mice. In contrast, in recipients that were adoptively transferred with CFSE-treated OT-I cells and subsequently challenged with OVA-pulsed APC, >50% of the CD8 T cells exhibited a reduced level of CFSE staining (Fig. 3*C*), demonstrating a normal and strong response by OT-I T cells upon encountering Ag in association with a professional APC. Thus, these data further indicate that naive OT-I T cells are nonresponsive to E.G7 tumor in vivo.

Because the OT-I T cells were adoptively transferred to mice with an established tumor, we considered that E.G7 might have anergized or otherwise suppressed activation of the transgenic T cells. To test this possibility, the proliferative responses in vitro by splenic and LN T cells to OVA peptide were assessed 4–7 days after adoptive transfer to normal or tumor-bearing mice. In both groups of mice, not only were the OVA-specific proliferative responses comparable, but the percentage of OT-I cells recovered from the spleen and LN were also very similar (Table I). This finding indicates that the failed anti-tumor response by OT-I T cells was not the result of Ag-specific nonresponsiveness induced by E.G7 in vivo. Collectively, these data indicate that the failed anti-tumor response by naive OT-I is largely due to immunological ignorance of OVA in the context of E.G7.

OT-I effector T cells generate an anti-tumor response

Although E.G7 as a solid tumor routinely failed to activate naive OT-I T cells in vivo, these cells remained as potential targets for immune effector cells, if such cells were successfully induced. To determine whether E.G7 was susceptible to OT-I effector cells, we tested whether the adoptive transfer of preactivated OT-I T cells generated an anti-tumor response. Before adoptive transfer, the OT-I cells were cultured for 3 days with OVA peptide and exogenous IL-2 and IL-4, and the effector cells were further expanded for 2 days by culture with only the cytokines. After this 5-day culture period, the OT-I cells exhibited potent CTL activity to E.G7, but not to EL4 (Fig. 4*A*). These effector CTL were adoptively transferred 5 days after s.c. injection of E.G7, and when compared with control mice, the growth of the tumor was delayed, especially at a relatively high dose (6×10^6) of OT-I effector cells (Fig. 4*B*). Thus, E.G7 was recognized by, and was accessible to, in vitro-generated OT-I $CD8^+$ effectors.

Because naive OT-I T cells were readily activated in vivo by OVA-pulsed APC (see Fig. 3), we next tested whether the in vivo induction of OT-I effector cells also induced anti-tumor

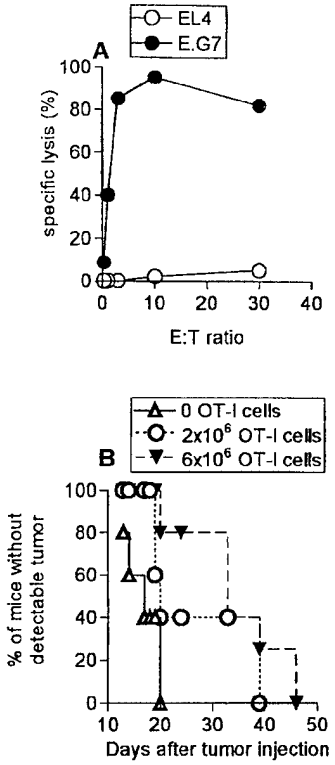

FIGURE 4. Activity of effector OT-I CTL cells. *A*, CTL activity in vitro as measured by ^{51}Cr-release assay against E.G7 and EL4. *B*, Anti-tumor activity of OT-I effector cells. Normal C57BL/6 mice were injected with E.G7 (1×10^6). Five days later, mice received the indicated number of T cells and tumor size recorded as described in Fig. 2. Effector OT-I CTL cells were generated as described in *Materials and Methods*. Data shown contain five mice/group and are representative of two experiments.

immunity. Naive OT-I T cells were adoptively transferred to mice 5 days after s.c. injection of E.G7, and 24 h later the mice received OVA-pulsed bone marrow-derived APC. As shown earlier, the tumor grew quickly in mice that received tumor in the presence of naive OT-1 (Fig. 5*A*). By contrast, coadministration of OT-I and OVA-pulsed APC resulted in a substantial delay in the progression of the tumor. In 40% of the mice, no tumor was detected 45 days after injection of E.G7, and several mice that were observed longer remained tumor-free on day 60. Importantly, there was no inhibition of growth when tumor-bearing mice received OVA-pulsed APC in the absence of OT-I T cells. This result indicates that the inhibition of tumor growth

Table I. *E.G7 tumor cells do not anergize OT-I cells*[a]

Expt.	Days after Adoptive Transfer	Cells from:	% OT-I[b]		cpm × 10^{3}[c]	
			Spleen	LN	Spleen	LN
1	7	Tumor-bearing mice	0.53 ± 0.03	1.39 ± 0.06	22.0 ± 3.3	88.3 ± 24.0
		Normal mice	0.67 ± 0.03	1.23 ± 0.03	24.3 ± 0.4	76.0 ± 16.6
2	4	Tumor-bearing mice	0.56 ± 0.08	1.50 ± 0.30	15.0 ± 4.2	92.0 ± 12.3
		Normal mice	0.55 ± 0.12	1.48 ± 0.21	13.5 ± 3.1	101.3 ± 7.4
3	4	Tumor-bearing mice	0.58 ± 0.02	0.94 ± 0.25	23.1 ± 4.0	71.5 ± 21.0
		Normal mice	0.60 ± 0.07	1.10 ± 0.15	21.0 ± 1.0	63.5 ± 15.4

[a] Normal C57BL/6 mice were injected with E.G7 (1×10^6). Five day later mice received 4×10^6 OT-I cells (experiment 1) and 2.5×10^6 OT-I cells (experiments 2 and 3). Spleen and LN were harvested at the indicated days, after adoptive transfer, and were subjected to analyses. Data shown contain three to four mice per group in each experiment.

[b] Determined by FACS analysis of three color positive cells for CD8, Vα2, and Vβ5.

[c] Determined by proliferation of spleen and LN after culture for 3 days with 1 nM OVA peptide.

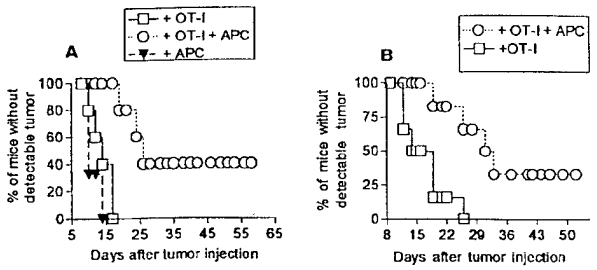

FIGURE 5. Peptide-pulsed APC induce anti-tumor response by naive OT-I cells. Normal C57BL/6 mice (*A*) or CD4-deficient mice (*B*) were injected with E.G7 (1×10^6). Five days later, the mice received naive OT-I T cells (3×10^6) and 1 day later peptide-pulsed APC (0.5×10^6) as indicated. The size of the tumor was recorded as described in Fig. 2. Data shown contain five mice per group and are representative of two experiments (*A*) and six mice per group (*B*).

is dependent upon the presence of the adoptively transferred transgenic T cells. Thus, after appropriate activation either in vitro or in vivo, E.G7 was susceptible to tumor (OVA)-specific OT-I effector cells.

To determine whether this anti-tumor response required $CD4^+$ T cells, we transferred OT-I T cells to tumor-bearing CD4-deficient mice (Fig. 5*B*). Similar to E.G7-bearing C57BL/6 normal mice, the growth of E.G7 was delayed in the $CD4^{-/-}$ recipient mice, if they received OVA-pulsed APC, and two of six mice remained tumor-free after 50 days.

The E.G7 tumor eventually grew in all mice that received in vitro-induced OT-I effector cells and in some mice that were stimulated with OVA-pulsed APC in vivo. The E.G7 cells were excised from one such mouse in each of the treatment groups and grown in culture for at least 7 days. These cells were then used as targets for OT-I CTL generated by in vitro culture. The E.G7 cells obtained from the mice treated with ex vivo-induced OT-I effector cells were nearly as good targets for OVA-specific CTL as the parental E.G7 (Fig. 6). Thus, the tumor outgrowth from this mouse appears to be the result of a failure of the adoptively transferred CTL to completely kill the tumor. However, E.G7 from the in vivo APC-treated mice were not lysed by the OVA-specific CTL, suggesting that tumor outgrowth in this case was caused by the selection of a tumor variant that escaped the effector OT-I CTL. In addition, ELISA analysis confirmed that these cells failed to secrete a detectable OVA level (data not shown). Collectively, these data raise the possibility that in vivo-induced effector T cells induced a more potent anti-tumor immune response than adoptive T cell immunotherapy.

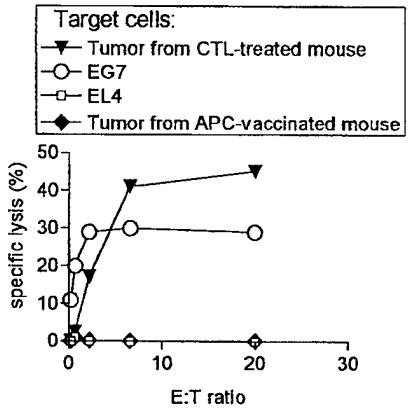

FIGURE 6. Lysis of tumor cells from OT-I-treated mice by OT-I effector cells in vitro. Tumors were excised from treated mice, as designated, grown in culture for at least 7 days, and labeled with ^{51}Cr to serve as targets for OT-I CTL (as described in *Materials and Methods*). ^{51}Cr-labeled E.G7 and EL4 served as positive and negative control targets, respectively.

Discussion

In the present study, we showed that a tumor-immune response could not be elicited in tumor-bearing mice after the adoptive transfer of a relatively high number of naive transgenic tumor-specific $CD8^+$ T cells. Importantly, as assessed at several different time points after adoptive transfer, the tumor-specific transgenic T cells were not activated to proliferate in both the spleen and the draining LN in the presence of the growing solid tumor. The failure to activate the T cells was not because the tumor anergized them, because OT-I T cells derived from tumor-bearing mice readily proliferated upon Ag challenge in vitro. Therefore, our data indicate that failed anti-tumor responses to E.G7 were due to immunological ignorance, i.e., a failure of the host immune system to recognize OVA, in this case in the context of a tumor cell, during the induction phase of the immune response.

It was surprising that the OT-I T cells remained ignorant of E.G7 when adoptively transferred in vivo considering the robust proliferative response of the T cells to irradiated E.G7 tumor cells in vitro. The activation of OT-I T cells in vivo has been shown to be dependent on Ag presentation by short-lived bone marrow-derived APC within the draining LN (24). Furthermore, when OVA is cell associated, e.g., in transgenic β-islet cells, activation of OT-I T cells is dependent upon cross-priming, i.e., the β-islet-associated OVA is ultimately processed by an exogenous class I pathway and presented to OT-I T cells by professional APC (24). Cross-priming is facilitated by either destruction of the Ag-containing cells and/or a high level of expression of the cell-associated Ag (25). In the case of β-islet cells, when cross-priming was not facilitated, the OVA-containing β-islets were ignored by OT-I T cells (26).

The failure of OT-I T cells to recognize E.G7 is not analogous to that described for OVA-containing β-islets, because E.G7 were able to directly present OVA to OT-I. It is likely that two factors promoted ignorance of E.G7. First, ignorance in our model appears to be at least in part the result of lack of contact between the OT-I tumor-specific T cells and OVA associated with the E.G7 tumor cells. Naive OT-I T cells appear not to readily migrate to the site of the tumor, preventing their direct activation by E.G7. Consistent with this hypothesis, so far we have not detected OT-I cells within the tumor site as determined by using CFSE-labeled OT-I cells and FACS analysis of the excised tumors (data not shown). Similarly, E.G7 does not obviously traffic to the spleen or the LN following s.c. or i.v. injections (data not shown). These observations suggest that OT-I T cells do not encounter E.G7 either at the site of the tumor or within the draining LN. Second, there is no indication that the E.G7 was able to cross-prime OT-I T cells by host APC. E.G7 produced relatively low levels of OVA, and minimal destruction of the rapidly growing E.G7 tumor is expected, especially early after injection of the tumor, conditions that would not favor cross-priming by the APC of the recipient mice. The observation that professional APC consistently activated OT-I T cells in the spleen and draining LN of E.G7-bearing mice demonstrates that ignorance to E.G7 is not due to a failure of the naive OT-I T cells to ultimately migrate to secondary lymphoid tissue, or to generalized immune suppression by E.G7, although we cannot exclude other means by which OT-I are ignorant of E.G7.

The value of using adoptive transfer of TCR- transgenic T cells to mice bearing tumors transfected with a model Ag is that this approach provides insight into the strength and duration of an anti-tumor T cell response. So far, two major outcomes have been observed in these types of studies. As we have seen for E.G7 as a solid tumor, tumor-specific TCR-transgenic T cells have been reported to be ignorant of L^d-transfected AG104A fibrosarcoma (27) and a glycoprotein of lymphocytic choriomeningitis virus after transfection into either Lewis lung carcinoma or the MC57G fibrosarcoma (17, 18). In the latter case, T cell ignorance required that the tumor be transplanted as a solid tumor fragment rather than s.c. injection of a single-cell suspension. These observations and our results suggest that T cell ignorance represents one important reason for failed anti-tumor immunity. Our data indicate that immunological ignorance may pertain to tumor-specific T cells bearing a high affinity for TCR, as OT-I T cells are extremely sensitive to OVA-peptide and proliferate to as little as 1 pM of peptide in vitro, a dose that is 1000-fold lower than that required to activate the lymphocytic choriomeningitis virus-specific TCR-transgenic T cells (17).

In other studies of the adoptive transfer of TCR-transgenic T cells to Ag-transfected tumors, an initial transient anti-tumor response was observed (14, 15, 28). In several cases, failed anti-tumor immunity was shown to be due to anergy of the tumor-specific T cells. This has been observed for both MHC class I- and II-restricted TCR-transgenic T cells. Interestingly, in the case of the MHC class I T cell response, OT-I T cells and E.G7 tumor cells also served as the model system (15). In that study, the E.G7 cells were injected into the peritoneal cavity 1 day after adoptive transfer of the OT-I T cells, which led to initial activation and proliferation of the OT-I T cells in vivo. Effective anti-tumor immunity failed in part due to CTLA4-mediated down-regulation of endogenous CD4 helper activity (29). This finding markedly contrasts with our result in which we found that OT-I T cells were ignorant of E.G7 as a solid tumor. We have compared the E.G7 subline maintained in our laboratory with that used by Srikant et al. (15) and found that our subline failed to activate OT-I after injection i.p. whereas the E.G7 subline used by Shrikant and coworkers activated OT-I when present as a solid tumor. This indicates that the different pattern of results is unlikely to be due to differences in experimental protocols and/or responses to a systemic vs solid tumor. Furthermore, both sublines of E.G7 produce similar levels of OVA. Therefore, it is most likely that these two cell lines express some intrinsic undefined difference, as the cells were independently passaged for a considerable period of time.

Although in our study naive OT-I T cells failed to inhibit the growth of E.G7, anti-tumor immune responses were elicited by OT-I effector cells, confirming that the OT-I T cells have sufficient affinity to specifically attack the developing E.G7 solid tumor. Effector OT-I T cells were generated either ex vivo in culture or by in vivo stimulation of the naive adoptively transferred OT-I T cells with peptide-pulsed bone marrow-derived APC. This illustrates that T cell ignorance can be overcome simply by proper Ag presentation of a tumor Ag. Furthermore, by using CD4-deficient mice, we demonstrated that this anti-tumor activity was independent of CD4 T cell help. Although many other studies clearly indicate a need for $CD4^+$ T cells for anti-tumor responses (30–33), our data, similar to that reported by Wick et al. (27), demonstrate that direct activation of CD8 T cells can be sufficient for potent anti-tumor immunity.

Several other studies have demonstrated that bone marrow-derived APC effectively inhibit tumor growth (17, 34), perhaps by activation of a population of ignorant tumor-specific T cells. It is interesting to note that so far we have only "cured" E.G7 with OT-I cells when the mice were immunized with OVA-pulsed APC. These findings raise the prospect that effective anti-tumor immunity may be facilitated by approaches that both increase the frequency of tumor-specific T cells and induce activation of such T cells by vaccination with tumor-Ag-containing APC.

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IL-2 during in vitro priming promotes subsequent engraftment and successful adoptive tumor immunotherapy by persistent memory phenotypic CD8$^+$ T cells[1]

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Abstract

Adoptive T cell tumor immunotherapy potentially consists of two protective components by the transferred effector cells, the immediate immune response and the subsequent development of memory T cells. The extent by which adoptively transferred CD8$^+$ CTL are destined to become memory T cells is ambiguous as most studies focus on the acute effects on tumor shortly following adoptive transfer. Here we show that a substantial fraction of the input CTL develop into memory cells that reject a subcutaneous tumor challenge. The use of exogenous IL-2 or a combination of IL-2 and IL-4, but not solely IL-4, during the ex vivo culture for the CTL inoculation was necessary for efficient development of CD8$^+$ memory T cells. Thus, an important component of adoptive immunotherapy using CTL is the production of CD8$^+$ antigen-specific memory cells which is primarily favored by IL-2 receptor signaling during ex vivo generation of the effector CTL.

Introduction

Adoptive immunotherapy represents a novel therapeutic option for tumors. This strategy involves removal of an individual's lymphocytes, generation of tumoricidal immune effector cells, and reinfusion of these effector cells into the tumor-bearing host. CTL are particularly attractive effector cells for this purpose, as they are specific and potent killers of all cells that bear the target antigen (1-3). Most studies on adoptive immunotherapy focus on the acute effects on tumor shortly following adoptive transfer. However, long-term protection against tumor recurrence requires that the infused CTL survive long after adoptive transfer, ideally without concomitant cytokine administration. In addition, it is desirable that these persistent donor T cells have characteristics of memory T cells, i.e. a very quick and intense response to the reappearance of tumor.

Differentiation into memory cells is in part regulated by cytokines (4, 5) and must result in

some antigen-specific T cells that are resistant to apoptosis (4, 6, 7). IL-7 and IL-15 are two cytokines that have been implicated in the regulation of homeostasis and survival of $CD8^+$ memory cells in vivo (5, 8-12). Comparatively less is known concerning how the cytokine environment during the initial priming of the naive T cells programs the resulting effector CTL to develop into memory cells. In most studies of adoptive immunotherapy, IL-2 has been typically employed for the ex vivo expansion of donor T cells that have been previously encountered tumor. The present study, therefore, was initiated to examine to what degree the ex vivo cytokine environment used to activate naive antigen-specific $CD8^+$ T cells subsequently favored their development into memory cells and protect against tumor activity in adoptive immunotherapy.

Materials and Methods

Animals. OT-I TCR transgenic mice (13) were maintained by breeding heterozygous OT-I TCR transgenic mice to wild-type C57BL/6J mice. The progeny were screened by PCR for the expression of the TCR transgene. All recipient mice were C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) and were used between 6-9 weeks of age.

Biological reagents. EL-4 is a thymoma derived from the C57BL/6 mouse ($H\text{-}2^b$). E.G7 cells consist of EL-4 cells transfected with OVA cDNA (14), and these were a gift from Dr. M. Bevan (University of Washington, Seattle, WA). These cell lines were maintained in complete medium (CM), consisting of RPMI 1640 containing 5% FCS, glutamine (30 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and β-mercaptoethanol (5×10^{-5} M). $OVA_{257\text{-}264}$ (SIINFEKL) (13) was synthesized by Research Genetics (Huntsville, AL). Cychrom-anti-CD8α, PE-anti-Vα 2-TCR, FITC-Vβ5.1,5.2-TCR, FITC-anti-CD8α (53.6.7), biotin-anti-Ly6C, biotin-anti-CD44 (Pgp-1), biotin-anti-CD62L (MEL-14), and biotin-anti-CD69 were purchased from BD Pharmingen (San Diego, CA). Biotin-anti-CD25 (7D4) was prepared in our laboratory. The PE-labeled MHC/peptide tetramer ($H\text{-}2K^b$/SIINFEKL) was provided by the NIAID MHC Tetramer Facility.

Cell culture. Unless indicated otherwise, OT-I CTL used for adoptive transfers were generated by the culture of OT-I splenocytes (1×10^6/well) in 24-well plates containing 1 ml of CM containing $OVA_{257\text{-}264}$ (1 nM), IL-4 (175 U/ml) and IL-2 (50 U/ml). After 3 days in culture, the cells were washed and re-cultured at 0.5×10^6 cells/well in 24-well plates containing 1 ml of CM without $OVA_{257\text{-}264}$ but with the same cytokines that were present during initial culture. After 2 additional days in culture, the cells were harvested and used for in vitro analyses or for adoptive transfer. CTL assays were performed as previously described (15) using ^{51}Cr-labeled E.G7 and EL-4 cells as targets. For T cell proliferation, spleen cells (2×10^5 cells/well) were cultured with the indicated concentration of $OVA_{257\text{-}264}$ in flat-bottom 96-well culture plates for 1-3 days. Each well was pulsed with 1 μCi of ^3H-thymidine and 5 hr later the cells were harvested with an automated harvester. The mean of triplicate values that varied by less than 10% was calculated. Data are expressed as Δcpm (i.e., cpm from experimental condition minus cpm from cultures containing culture medium alone).

FACS Analysis. Cells were stained with the various antibody conjugates as previously described (15). Between 50,000-100 ,000 events were collected and analyzed for each sample using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) and CellQuest software.

Adoptive immunotherapy. OT-I CTL were injected via the tail vein in 0.5 ml HBSS into normal C57BL/6J mice. Control mice for the adoptive transfers received only HBSS. The proportion

of input cells that consisted of OT-I CTL was determined by staining for CD8α, Vα2-TCR and Vβ5.1,5.2-TCR, and this information was used to calculate the number of OT-I CTL adoptively transferred. E.G7 or EL-4 cells (1×10^6) were injected subcutaneously in 0.2 ml HBSS at the midline of the lower abdomen 21-28 days after adoptive transfer of OT-I CTL. The tumor cells were freshly thawed within 6 days of inoculation. Tumor cell growth was recorded over time. Detectable tumor was considered to be >0.5 cm^2. Tumor-free survival curves were compared by log-rank test. A p-value of < 0.05 was considered significant.

Results

Persistence, phenotype, and function of adoptively transferred CTL. We recently reported that naive T cells from the OT-I T cell receptor (TCR) transgenic mice, specific for an OVA peptide, $OVA_{257-264}$, in the context H-$2K^b$, failed to recognize and respond to the OVA-transfected E.G7 thymoma, i.e. were immunologically ignorant, after adoptive transfer into mice bearing E.G7 as a solid tumor (15). However, the adoptive transfer of OT-I effector cells, generated in short-term cultures, inhibited the growth of E.G7 in a dose-dependent manner (15). In the current study we have continued to use this model system to study the ability of in vitro-derived OT-I CTL effector cells to develop into memory cells in vivo and to then function in anti-tumor immunity.

Our past work indicated that the generation of primary tumor-specific CTL in vitro was favored by culture with exogenous IL-2 and IL-4 (16). Therefore, splenocytes from naive OT-I transgenic mice were stimulated with $OVA_{257-264}$ in the presence of exogenous IL-2 and IL-4 for 3 days and expanded in these exogenous cytokines for an additional 2 days. On the fifth day, greater than 90% of cells consisted of $CD8^+$ cells that co-expressed Vα2 and Vβ5, characteristic of the OT-I TCR (not shown). FACS analysis revealed that these activated cells typified effector T lymphocytes, i.e. increased size, up-regulated expression of CD69, CD25 and CD44, and down-regulated expression of CD62L and Ly-6C (Fig. 1). Furthermore, these effector cells exhibited potent and specific CTL activity to OVA-expressing targets (Fig. 4C).

These CTL effector cells were adoptively transferred to syngeneic C57BL/6 recipient mice. The number of OT-I CTL effector cells that persisted 21-28 days after adoptive transfer was assessed by the fraction of cells that were "triple-stain positive" for CD8, Vα2, and Vβ5 (Fig. 2A). This analysis indicated that the number of "triple-stain positive" cells in the spleen and lymph nodes was proportional to the number of cells administered (Fig. 3A and 3B). In each animal, the spleen, two axillary lymph nodes, and two inguinal lymph nodes were analyzed. In sum, in animals which received 10×10^6 CTL, the mean number of "triple-stain positive" lymphocytes recovered from a pool of these organs was $4.4 \pm (1.6) \times 10^6$ (Fig. 3B). Given that the mean number of "triple-stain positive" cells in control mice is $0.4 \pm (0.2) \times 10^6$, the estimated recovery of OT-I cells in these organs is 40% of the input cells. If one were to extrapolate this number to the entire peripheral lymph tissue, about 68% of the OT-I cell persisted at this time, assuming 25 lymph nodes in a mouse containing an equal number of OT-I cells in each node.

FACS analysis revealed that these "triple-stain positive" cells were smaller than the adoptively transferred CTL as assessed by forward light scatter (Fig. 1). The phenotype of the OT-I cells was further characterized for other surface markers by gating on $CD8^+$ and Vα2+ cells (Fig. 1). Unlike the in vitro generated OT-I CTL effector cells, the in vivo retained cells no longer expressed elevated

levels of CD69 or CD25, but Ly6C was substantially upregulated. In addition, CD44 remained upregulated while CD62L reverted to a higher degree of expression. This cell surface phenotype is consistent with that attributed to memory T cells (17-19). In 3 mice tested at 70-75 days after adoptive transfer, "triple-stain positive" (Fig. 2B) or major histocompatibility complex (MHC)-tetramer-positive (Fig. 2C) OT-I T cells were readily detected with a cell surface phenotype identical (not shown) to that observed at 21-28 days after adoptive transfer. All of these features except the elevated Ly6C expression were detected as early as 7 days after adoptive transfer (not shown). At 21-28 days after adoptive transfer, 5 of 5 mice tested expressed a high level of Ly6C. However, in two mice tested at earlier time points, the high Ly6C expression was not seen, suggesting that this aspect of the phenotype appears relatively late.

Memory CD8 T cells often show more rapid proliferation to a lower concentration of antigen when compared to naive T cells (18-21) and retain CTL activity (22-24). When compared to naive T cells, the recovered adoptively transferred "memory" OT-I T cells proliferated to an approximately 10-fold lower concentration of $OVA_{257-264}$ (Fig. 4A) and more rapidly (Fig. 4B). With respect to CTL activity, the level of cytotoxicity was proportional to the number of OT-I $CD8^+$ cells that persisted in the spleen (Fig. 4D). Thus, collectively these data demonstrate that 21 days after adoptive transfer, the OT-I effector cells also exhibited functional properties characteristic of memory $CD8^+$ T cells.

To assess the anti-tumor activity of the persistent OT-I T cells, mice received E.G7 subcutaneously 21-28 days after adoptive transfer of the OT-I CTL. Unlike control-treated animals, mice that received OT-I CTL were generally resistant to E.G7 tumor inoculation (Fig. 5). This protection was specific to OVA-expressing tumors, as these mice did not mount an anti-tumor response when inoculated with the parental EL-4 thymoma. Furthermore, mice (n=2) adoptively transferred with OT-I CTL, that rejected a primary challenge with E.G7, remained tumor-free after a second challenge with E.G7 when re-inoculated 56 and 66 days after the first inoculation (not shown). Thus, long-lasting protective tumor-specific immunity was not prevented by the initial response by the persistent OT-I T cells.

Development of OT-I memory T cells and anti-tumor response is dependent upon the cytokines used to generate the effector cells. Recent data suggest that generation of CTL in the presence of IL-4 enhances their survival following adoptive transfer (25). Therefore, we examined the effect of IL-2 and IL-4 during the in vitro culture on the long-term fate and function of OT-I CTL. In these experiments, the OT-I T cells were stimulated with a lower dose (0.1 nM) of $OVA_{257-264}$ as this was found to be necessary for optimal cytokine expansion of these CTL (data not shown).The proportion of cells staining for CD8, Vα2 and Vβ5 did not differ significantly between conditions and was typically 87-95% of the cells (not shown). When compared to naive OT-I T cells (see Fig. 1), all effector cells upregulated CD69, CD25 and CD44 while the levels of CD62L and Ly-6C were down-regulated regardless of the exogenous cytokine(s) added during the priming cultures (Fig. 6 and data not shown). However, the phenotype of these effector cells were not identical as cells cultured in only exogenous IL-4 exhibited lower levels of CD25 and CD44. The former result likely represents the lack of endogenous IL-2 to upregulate CD25 (26). Intragroup variation was sometimes noted for the level of expression of the early activation antigen CD69 and Ly-6C irrespective of the exogenous cytokine added to the cultures. Furthermore, although all effector groups displayed strong CTL activity to E.G7, effector cells generated in the presence of IL-4 or both

IL-2 and IL-4 showed stronger activity than cells expanded with only IL-2 (Fig. 6B).

These effector OT-I CTL were adoptively transferred into C57BL/6 mice and their persistence in the spleen and lymph nodes was assessed 21–28 days later. The largest number of OT-I cells was detected in recipients of CTL generated with either IL-2 or a combination of both IL-2 and IL-4 (Fig. 7). In contrast, a markedly lower number of "triple-stain positive" cells was detected in recipients of CTL generated in IL-4. The use of a higher dose of IL-4 did not improve the degree of persistence of the OT-I. These findings were confirmed by staining for the donor cells with MHC-peptide tetramer (Fig. 7). These data indicate that stimulation with IL-2 during the generation of CTL optimally promotes the survival of these cells upon adoptive transfer in vivo.

FACS analysis revealed that the phenotype of the persistent OT-I CTL generated in either IL-2 or both IL-2 and IL-4 was typical of a memory phenotype, i.e. $CD25^{low}$, $CD44^{high}$, and $Ly6C^{high}$ (Fig. 8). By contrast, only a small proportion of the persistent OT-I cells generated in IL-4 expressed high levels of CD44 and Ly6C (Fig. 8). This finding in conjunction with the lower yield of persistent OT-I cells (Fig. 7) indicate that there are 4-6 fold fewer cells with a memory phenotype when the naive OT-I were solely primed ex vivo with antigen and exogenous IL-4.

To examine antigen-specific responsiveness, spleen cells from adoptively transferred mice were stimulated with $OVA_{257-264}$ in vitro. In general, the magnitude of the resulting proliferative response was proportional to the number of donor cells identified in the spleen. However, this relationship was not linear, as the magnitude of this response by the splenocytes in recipients of OT-I cells generated in IL-4 was disproportionately low (Fig.9A).

At 21–28 days after adoptive transfer, recipients of OT-I CTL generated in each cytokine condition were inoculated subcutaneously with E.G7 cells. When compared to untreated cells, all treatment groups exhibited a significant increase in tumor free survival (IL-2, $P < 0.001$; IL-4, $P < 0.001$, IL-2/IL-4, $P < 0.006$; $IL\text{-}4^{HI}$, $P = 0.001$) (Fig. 9B). It is noteworthy that the IL-4 generated OT-I effector cells developed a therapeutic effect even though these cells inefficiently persisted and developed a memory phenotype. Thus, these residual cells were still competent to affect E.G7 growth. Between the treatment groups, the difference in tumor-free survival between mice that received OT-I effector cells generated with IL-2 and recipients of CTL generated in IL-2/IL-4 and IL-4 (50 U/ml) was not significant. However, the greater tumor free survival in mice which received OT-I effector cells generated in IL-2 when compared to mice which received CTL primed in a high dose of IL-4 (175 U/ml) was statistically significant ($P = 0.04$). The greater therapeutic efficacy of OT-I generated in IL-2 may be related to its ability to optimally promote differentiation of the effector CTL into persistent memory phenotypic cells, as these effector cells exhibited the weakest CTL activity upon adoptive transfer to tumor-bearing mice (Fig. 6B).

Discussion

Many previous studies have demonstrated that protective memory responses against tumor cells occur in vivo. In these cases, the memory response is typically demonstrated after either naive or effector tumor-specific T cells have first elicited a protective primary anti-tumor response. For adoptive immunotherapy, it has been difficult to ascertain whether the memory response is dependent upon encountering tumor antigens in vivo or if it reflects an intrinsic potential of the transferred effector cells. Based on both phenotypic and functional characteristics, our data support the notion

that a substantial portion of adoptively transferred CTL differentiate into memory T cells that are then competent to reject a tumor challenge. At 21-28 days after adoptive transfer, the persistent OT-I T cells expressed a cell surface phenotype of memory cells, i.e. $CD44^{high}$, $CD62L^{high}$, $Ly\text{-}6C^{high}$, $CD25^{neg}$, and $CD69^{neg}$ (17-19). Furthermore, unlike naive OT-I T cells, these persistent OT-I cells expressed intrinsic CTL activity and exhibited rapid proliferation to a relatively low dose of $OVA_{257\text{-}264}$ upon in vitro challenge. These functional properties have been reported to be features that distinguish memory T cells (6, 18-21, 24).

When studying cell populations thought to represent memory T cells, it is imperative that the cells under study be distinguished from chronically stimulated effector T cells that persist secondary to antigen. Even cells present several months after adoptive transfer may display characteristics more consistent with effector cells (27). Identification of $CD8^+$ memory cells is also problematic in some models in which T cells are adoptively transferred to lymphopenic mice. In this situation naive $CD8^+$ T cells acquire the phenotypic and functional characteristics of memory T cells in the absence of antigen by the process of homeostatic proliferation (28-30). In our model we have avoided potential complications due to either antigen or homeostatic proliferation by removing extraneous antigen by washing the effector cells several days prior to adoptive transfer to mice with a normal pool of lymphocytes.

A number of studies have documented that exogenous cytokines influence the magnitude and quality of the resulting $CD8^+$ effector CTL generated during in vitro culture (16, 31-34). More recently we demonstrated an essential role for cytokines for the generation of effector CTL. In the absence of IL-2R and IL-4R signaling in vitro, TCR-activated $CD8^+$ T cells failed to differentiate into CTL, in part due to lack of expression of granzyme B, and exhibited limited proliferative capacity (3-4 cell divisions) prior to apoptosis by the vast majority of the cells (35). Comparatively little is known concerning the long-term consequences of these culture conditions on CTL effector cells in vivo. Our findings demonstrate a critical role for IL-2 during in vitro generation of the effector CTL for the optimal in vivo persistence of CTL with characteristics of memory T cells. Analysis of allospecific T cells in autoimmune prone IL-2-deficient mice and an antigen-specific $CD8^+$ cytotoxic T cell line have suggested that IL-2 favors the development of T memory cells (36, 37). Our data directly implicate signaling through the IL-2R for development of CD8 T memory cells from naive normal antigen-specific precursor T cells. In this regard it is important to note that the sole use of exogenous IL-4 resulted in production of optimal numbers of CTL. However, upon adoptive transfer, these effector cells did not readily persist or express a memory phenotype. These IL-4 driven effector CTL were correspondingly less effective in anti-tumor immunity.

Several other studies have reported findings consistent with our results. In a model of viral infection, Aung et al. (38) showed that the presence of IL-4 at the time of activation diminishes the expansion of viral antigen-specific $CD8^+$ T cells and inhibits the development of $CD8^+$ T cell memory. Similarly, Villacres and Bergmann (39) demonstrated that the absence of IL-4 at the time of activation of CTL increases the frequency of $CD8^+$ memory cells 8 weeks later and enhances the effector functions of these memory cells. In our experiments, co-culture of T cells with exogenous IL-2 alone or both IL-2 and IL-4 resulted in enhanced long-term survival and differentiation to the memory phenotype suggesting that signal transduction through the IL-2R is sufficient to promote memory CTL upon adoptive transfer in vivo. This result also indicates that IL-4 does not actively prevent engraftment of CTL that persist with a memory phenotype. However, our experiments do

not rule out that signals through other cytokine receptors or T cell surface proteins might also favor the engraftment and espression of the memory phenotype by $CD8^+$ effector cells. Furthermore, our data do not address whether IL-2 also functions in vivo to promote memory CTL. IL-15, whose receptor also utilizes IL-2Rβ and γc for signaling, has been shown to play an important role in the homeostasis of $CD8^+$ memory cells (5, 9-11) and is also a candidate to promote memory CTL in vivo.

Although IL-2 and IL-4 redundantly function as T cell growth factors and promote differentiation into CTL in vitro (35, 40, 41), our data suggest that IL-2 is mandatory for promoting memory CTL. This finding was somewhat surprising because Huang et al. (25) reported that $CD8^+$ T cells activated in the presence of IL-4 in vitro persisted to a greater degree after adoptive transfer than CTL activated in IL-2. These persistent cells expressed high levels of CD44 and produced large amounts of IFNγ following re-exposure to peptide suggesting that they consisted of memory cells. However, it should be emphasized that while these investigators initially stimulated the $CD8^+$ T cells in either IL-2 or IL-4 for 3 days, all cells were expanded in IL-2 for an additional 2 days prior to adoptive transfer. Thus, analogous to our experimental design, these T cells also received signaling through the IL-2R prior to adoptive immunotherapy which we believe likely contributed to promoting memory CTL in vivo.

This study reveals an unappreciated potential value of adoptive anti-tumor immunotherapy. Besides the initial anti-tumor response by effector CTL, in vitro expansion using IL-2 promotes the production of memory $CD8^+$ T cells which may favor long-term protection against tumor recurrence. Furthermore, adoptive immunotherapy with effector CTL has been shown to overcome immunological ignorance (15), a phenomenon by which naive tumor-specific T cells simply ignore tumor-associated antigen. Nevertheless, in spite of these beneficial features, adoptive immunotherapy with ex vivo IL-2 expanded tumor-infiltrating lymphocytes that contain CTL is often unsuccessful in cancer patients. In addition to tumor-induced immunosuppression and outgrowth of tumor antigen escape variants, growth factor deprivation is a problem after adoptive transfer, particularly when CTL have been generated and expanded over longer periods of time (1). In the model described herein, unlike in most therapeutic protocols, large numbers of effector cells were generated over a much shorter time frame. Therefore, successful adoptive T cell therapy may be linked to rapid production of effector T cells using the appropriate cytokines during the ex vivo culture.

Acknowledgments

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Footnotes

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Figure legends

Fig. 1. Phenotypic properties of naive, effector, and adoptively transferred OT-I T cells. Cell surface phenotype by FACS analysis. Splenocytes from a naive OT-I mouse were directly subjected to FACS analysis or activated with $OVA_{257-264}$, IL-4 and IL-2 as described in the Methods. On the fifth day, the OT-I effector cells were subjected to FACS analysis and administered to normal syngeneic recipients. Splenocytes from the recipient mice were then harvested 28 days later for FACS analysis. OT-I $CD8^+$ T lymphocytes were identified by gating for expression of CD8 and Vα2-TCR and then analyzing for the expression of the indicated markers (shaded histograms). Negative controls are represented by unshaded histograms. Data are representative of 5 experiments.

Fig. 2. FACS identification of adoptively transferred OT-I $CD8^+$ T cells. Spleen cells were analyzed for OT-I T cells 23 (A) and 74 days (B, C) after control injection of HBSS or adoptive transfer of OT-I CTL (10×10^6) into C57BL/6 mice, as listed above each dot plot. OT-I T cells were enumerated by triple staining with antibodies to CD8, Vα2-TCR and Vβ5.1,5.2-TCR (A, B) ("triple stain positive) or double staining with anti-CD8 and the H-$2K^b$/SIINFEKL tetramer. The fraction of the gated cells fluorescent positive for the indicated surface markers are listed in the

dot plots and histograms. Data are representative of 3–5 mice per group.

Fig. 3. Persistence of adoptively transferred OT-I CD8$^+$ T cells. A) Number of "triple-stain positive" cells expressed as a percentage of all CD8$^+$ cells. B) Number of "triple-stain positive cells", calculated as the product of the total number of lymphocytes recovered in the spleen plus 4 lymph nodes and the percentage of "triple-stain positive" cells. The indicated numbers of OT-I CTL were adoptively transferred and 21-28 days later the spleen and lymph nodes (inguinal and axillary) were analyzed by FACS. Each group consisted of 3–5 recipient mice that were individually analyzed.

Fig. 4. Functional properties of naive, effector, and adoptively transferred OT-I T cells. A, B) Proliferative responses by naive and persistent adoptively transferred OT-I T cells. Dose-response to $OVA_{257-264}$ 48 hr after culture initiation. B) Time-course of proliferative response using 100 pM $OVA_{257-264}$. Persistent OT-I T cells were analyzed 21-28 days after adoptive transfer. Splenocytes from a naive OT-I mouse were mixed with normal C57BL/6 splenocytes to contain a fraction of OT-I cell equivalent to that in the spleen from the adoptively transferred recipient (typically approximately 4%). C, D) CTL activity by ex vivo effector and persistent adoptively transferred OT-I T cells. C) OT-I effector cells were prepared as described in the Materials and Methods and directly tested for CTL activity against E.G7 or EL4 targets. D) CTL activity by splenocytes from adoptively transferred mice that received the indicated number of OT-I CTL 21-35 days previously. Data shown represent specific CTL activity against E.G7 target cells at an effector-to-target ratio of 100:1. Data are representative of 3 experiments (A-C) or 3 mice per group (D).

Fig. 5. Anti-tumor activity of memory OT-I T cells. 21-28 days after adoptive transfer of 10 x 10^6 OT-1 CTL (prepared as described in the Methods), recipient mice were inoculated with 1 x 10^6 E.G7 or EL-4 cells, s.c., as indicated. As a control, some mice received HBSS rather than OT-I CTL. Data are represented as tumor-free survival for $\geq$6 mice/group.

Fig. 6. Phenotype and function of OT-I effector cells generated with IL-2 and/or IL-4. OT-I splenocytes were stimulated with $OVA_{257-264}$ (0.1 nM) and IL-2 (50 U/ml) and /or IL-4 (50 U/ml), or IL-4^{HI} (175 U/ml) as described in the Methods for 5 days. A. FACS analysis prior to adoptive transfer. Staining for the indicate markers (dark line) or negative controls (light line) are shown. B) CTL activity just prior to adoptive transfer. Data are representative of 2 experiments.

Fig.7. The persistence of adoptively transferred OT-I CTL generated with IL-2 and/or IL-4. OT-I effector cells were generated as described in the legend to Fig. 6 and then 10 x 10^6 effector cells were adoptively transferred to normal C57BL/6 mice. The number of persistent donor cells was determined 21-28 days after adoptive transfer as estimated by the product of the number of lymphocytes harvested in spleen and 4 lymph nodes and the fraction of "triple-stain positive" cells or the fraction of cells staining with anti-CD8 and H-2K^b/SIINFEKL tetramer. Data were derived from 3 experiments.

Fig. 8. The phenotype of persistent adoptively transferred OT-I CTL generated with IL-2 and/or IL-4. Expression of CD44 and Ly6C (dark line) or negative controls (light line) by persistent OT-I T cells gated for expression of both CD8 and Vα2-TCR. OT-I effector cells were adoptively transferred as described in the legend to Fig. 7 and recipient spleen cells were analyzed 21-28 days after adoptive transfer. Data are representative of 3 experiments

Fig. 9. Functional activity of persistent adoptively transferred OT-I CTL generated with IL-2 and/or IL-4. OT-I effector cells were adoptively transferred as described in the legend to Fig. 7. A) Proliferative response by splenocytes from adoptively transferred mice that received OT-I CTL 21-28 days previously. Data are derived from 3 separate experiments. B) Tumor-free survival of recipient mice that received OT-I CTL. 6-7 mice/group were inoculated with E.G7 tumor 21-28 days after adoptive transfer. Day 0 refers to the day when the mice received E.G7.

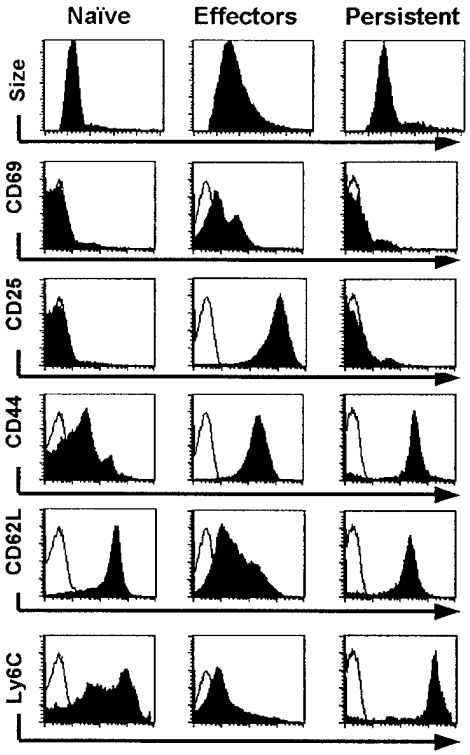


Figure 1

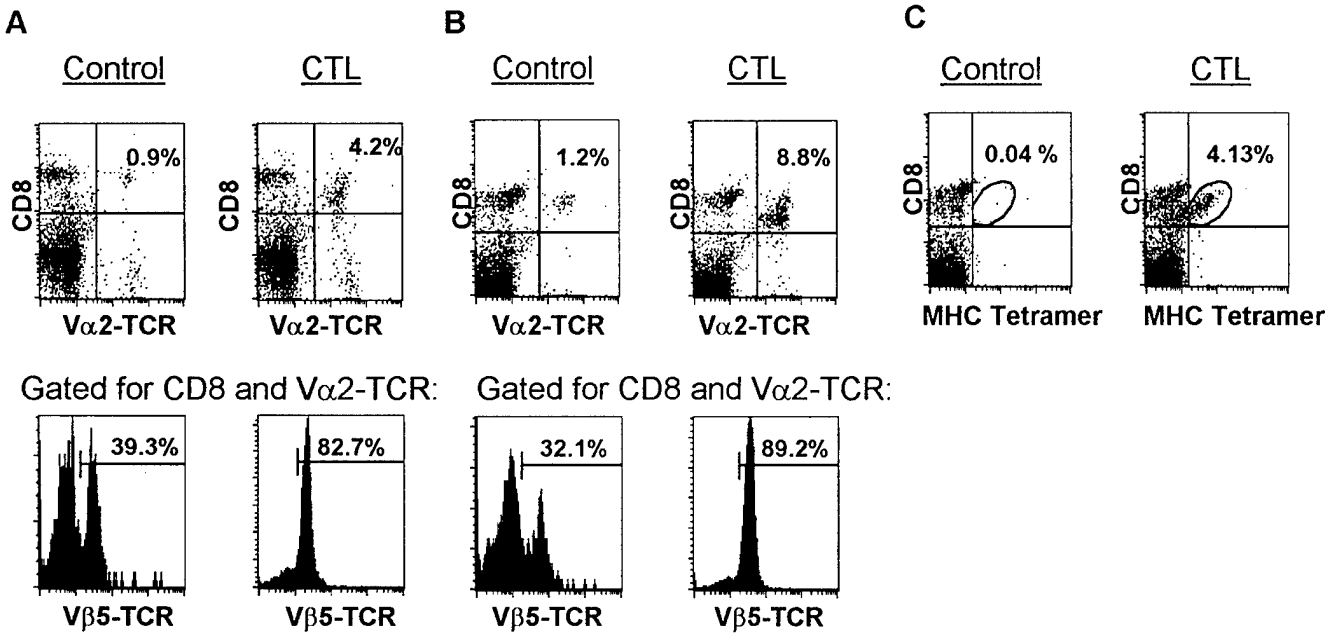
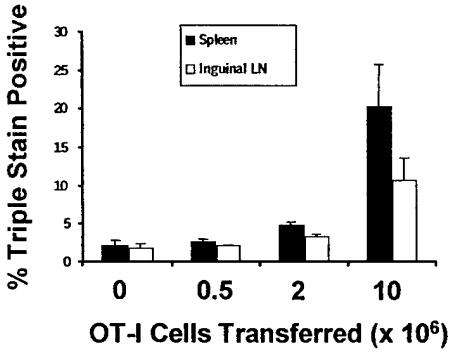

A
Control
CTL
0.9%
4.2%
CD8
Vα2-TCR
B
Control
CTL
1.2%
8.8%
CD8
Vα2-TCR
C
Control
CTL
0.04 %
4.13%
CD8
MHC Tetramer
Gated for CD8 and Vα2-TCR:
39.3%
82.7%
Vβ5-TCR
Gated for CD8 and Vα2-TCR:
32.1%
89.2%
Vβ5-TCR

Figure 2

A


% Triple Stain Positive
Spleen
Inguinal LN
30
25
20
15
10
5
0
0
0.5
2
10
OT-I Cells Transferred (x 10^6)

B

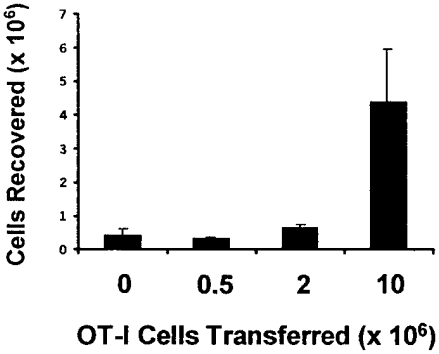

Cells Recovered (x 10^6)
7
6
5
4
3
2
1
0
0
0.5
2
10
OT-I Cells Transferred (x 10^6)

Figure 3

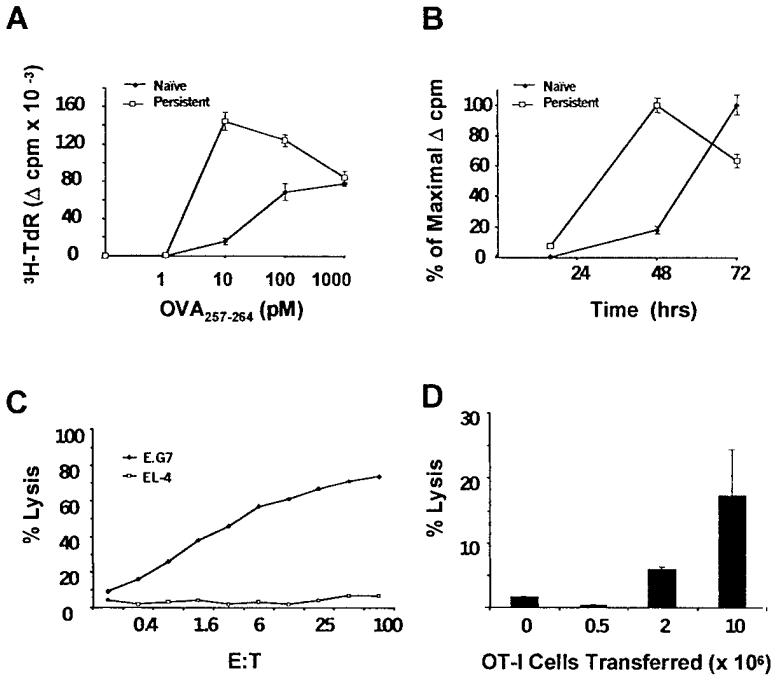

A
B
C
D
Naïve
Persistent
^{3}H-TdR (Δ cpm x 10^{-3})
160
120
80
40
0
1
10
100
1000
OVA$_{257-264}$ (pM)
% of Maximal Δ cpm
100
80
60
40
20
0
24
48
72
Time (hrs)
% Lysis
E.G7
EL-4
100
80
60
40
20
0
0.4
1.6
6
25
100
E:T
30
20
10
0
0.5
2
10
OT-I Cells Transferred (x 10^6)

Figure 4

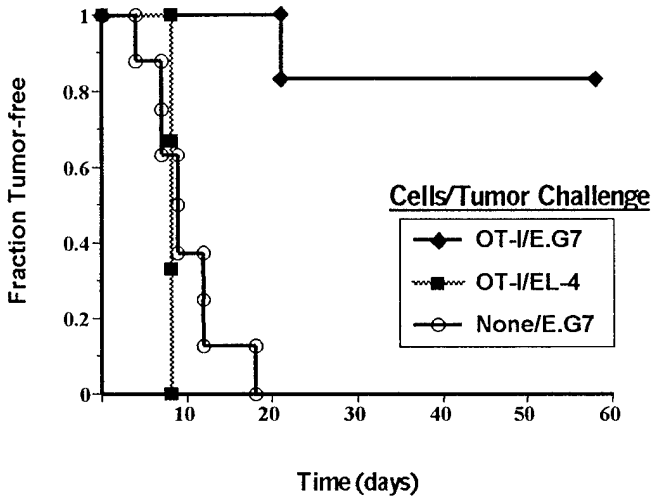

Fraction Tumor-free
1
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
Time (days)
Cells/Tumor Challenge
OT-I/E.G7
OT-I/EL-4
None/E.G7

Figure 5

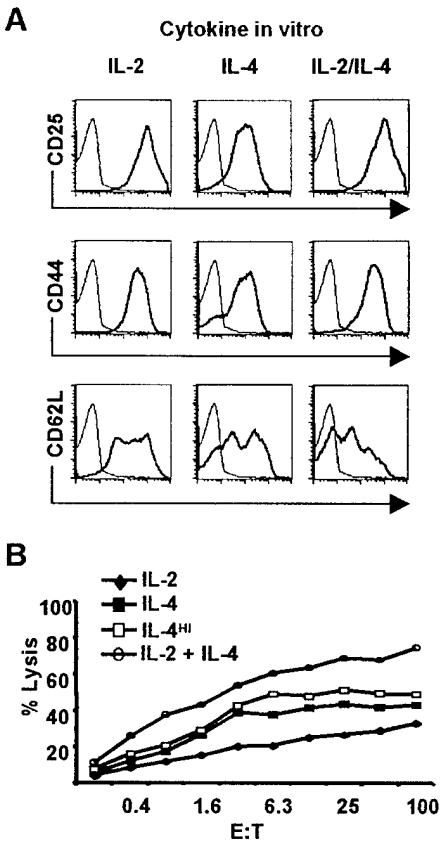

A
Cytokine in vitro
IL-2
IL-4
IL-2/IL-4
CD25
CD44
CD62L
B
IL-2
IL-4
IL-4HI
IL-2 + IL-4
% Lysis
100
80
60
40
20
0.4
1.6
6.3
25
100
E:T

Figure 6

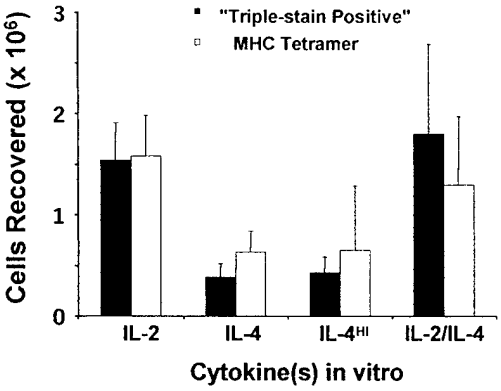

"Triple-stain Positive"
MHC Tetramer
Cells Recovered (x 10^6)
3
2
1
0
IL-2
IL-4
IL-4HI
IL-2/IL-4
Cytokine(s) in vitro

Figure 7

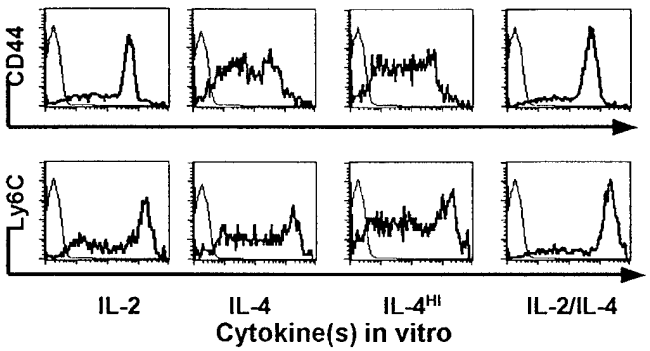


Figure 8

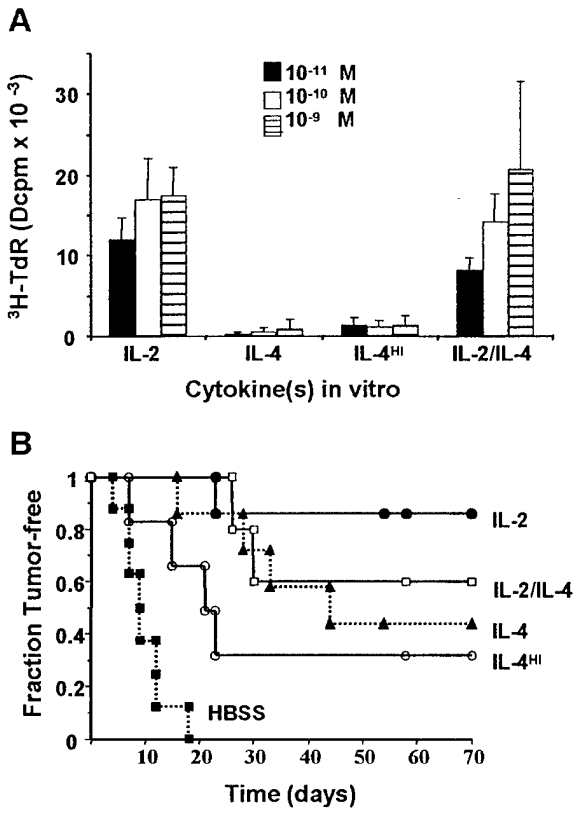

A
10^{-11} M
10^{-10} M
10^{-9} M
^{3}H-TdR (Dcpm x 10^{-3})
30
20
10
0
IL-2
IL-4
IL-4^{HI}
IL-2/IL-4
Cytokine(s) in vitro
B
Fraction Tumor-free
1
0.8
0.6
0.4
0.2
0
IL-2
IL-2/IL-4
IL-4
IL-4^{HI}
HBSS
10
20
30
40
50
60
70
Time (days)

Figure 9